Density-Enhanced Protein Tyrosine Phosphatase 1 (DEP-1) Regulation of Epithelial Cell Adhesions

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Abstract

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(Under the direction of Keith Burridge)

Cell-cell adhesions are critical to the development and maintenance of multicellular organisms. Increased tyrosine phosphorylation of junctional proteins has been associated with promoting disassembly of protein complexes at junctions and reducing cell-cell adhesions. Levels of tyrosine phosphorylation reflect the balance between protein-tyrosine kinase (PTK) and protein-tyrosine phosphatase (PTP) activity. DEP-1 is a receptor PTP which localizes to cell-cell adhesions and has been implicated in regulating phosphorylation of junctional proteins. The catalytically dead substrate-trapping mutant of DEP-1 (DEP-1 D/A) was used to identify additional substrates at cell-cell junctions. Members of the tight junction, occludin and ZO-1, were found to be substrates of DEP-1. DEP-1 D/A was not only able to bind these proteins in a tyrosine-phosphorylation dependent manner but wild type DEP-1 was able to dephosphorylate them. Occludin and ZO-1 interactions with DEP-1 were mediated through binding to the catalytic domain of DEP-1 and not by other protein-protein interaction motifs examined and appear to be specific to DEP-1. Over-expression of DEP-1 increased transepithelial electrical resistance in confluent epithelial monolayers and also reduced paracellular flux of FITC-dextran following a calcium switch. In addition, FAK

and paxillin were also identified to be substrates of DEP-1 and indicate that DEP-1 could be regulating integrin adhesion-mediated signaling as well. Future work will focus on mapping the occludin residues necessary for DEP-1 interaction and will help to clarify potential signaling pathways and kinases upstream of DEP-1 activity. By controlling phosphotyrosine levels of tight junction proteins and perhaps focal adhesion proteins, DEP-1 may play a role in regulating permeability and junction formation in epithelial cells.

To my parents Lynn and Linda Sallee, who have always believed in me and supported me

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Table of Contents

List of Tables

List of Figures

Abbreviations

AJ	adherens junction
DEP-1	density enhanced phosphatase 1
ECM	extracellular matrix
FAK	focal adhesion kinase
FITC	fluorescein isothiocyanate
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GST	glutathione S-transferase
HEK	human embryonic kidney
LMW-PTP	low-molecular weight protein tyrosine phosphatase
MAM	meprin/A5/RPTPμ
MDCK	Marin-Darby canine kidney
PDZ	PSD-95/DlgA/ZO-1
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PDGF	platelet derived growth factor
ROS	reactive oxygen species
RPTP	receptor protein tyrosine phosphatase
SAP-1	stomach cancer-associated PTP-1
SFK	Src family kinase
TEM	transendothelial migration

TER	transepithelial electrical resistance
TJ	tight junction
VE-cadherin	vascular endothelial cadherin
VE-PTP	vascular endothelial protein tyrosine phosphatase
ZO-1	zonula occludens-1

Chapter 1:

Introduction

The regulation of cell-cell adhesion is important in many physiological processes. In the immune system, regulation of endothelial cell-cell junctions is critical for passage of white blood cells from the blood to underlying tissue that may be damaged or infected. If disassembly or reassembly is disregulated, you are left with either a weak immune response or a constitutive response associated with several inflammatory disorders such as arthritis. Another example of disregulated junctions is in epithelial-to-mesenchymal transition (EMT). As cells become transformed, they can lose expression of cellular adhesion molecules, resulting in loss of adhesion to matrix and to adjacent cells. These transformed cells now have increased mobility and are able to travel to different sites (i.e. metastasis). One way in which cell-matrix and cell-cell adhesions can be regulated is through the control of tyrosine phosphorylation levels of proteins at points of cell adhesions. Protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) are responsible for balancing phosphotyrosine levels. The focus of my work has been on the role of PTPs, DEP-1 in particular, in regulating cell-cell adhesions. In the next several pages I will give you an overview of the regulation of cellular adhesions by classical PTPs.

Regulation of cell adhesion by protein tyrosine phosphatases

Cell adhesion and migration are two tightly coupled processes critical to normal development and physiology. Two types of adhesion are usually distinguished: adhesion of cells to the underlying extracellular matrix (ECM) and adhesion between adjacent cells. Adhesions are more than simple physical links to the matrix or to other cells; they are also sites where signals are initiated, allowing cells to monitor their immediate environment. Prominent among the signaling pathways that emanate from adhesion sites are those involving protein tyrosine phosphorylation. Tyrosine phosphorylation is a major post translational modification that regulates many signal transduction pathways involved with proliferation and differentiation, and in communication between adjacent cells and cell-matrix interactions. The differential tyrosine phosphorylation of cell adhesion molecules and their associated proteins is one means of altering the assembly and stability of adhesions. Phosphotyrosine levels reflect the balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).

PTPs were discovered several years after PTKs and have been studied less extensively. However, the number of genes encoding PTPs rivals that of PTKs, suggesting that the functions of PTPs may be just as complex (1). In addition, the diversity of phenotypes in knockout mice lacking various PTP genes demonstrates that many PTPs have non-redundant functions. Although PTPs were first believed to behave as "housekeeping" proteins, terminating signaling pathways initiated by PTKs, it is now appreciated that PTPs can activate kinases and other enzymes by removing inhibitory phosphates, thus playing a more active role in signaling pathways. Several families of PTPs have been identified, including classical PTPs, dual specificity PTPs, myotubularins, PTEN-related PTPs and aspartic acid-based PTPs (2). I will focus primarily on the classical PTPs and their functions

in cell-matrix and cell-cell adhesions. Classical PTPs contain a highly conserved catalytic domain with a critical cysteine sulfhydryl in the catalytic site. They show considerable diversity in their other domains, allowing for variations in binding partners, localization, and function. In humans, 38 classical PTPs have been identified and these fall into two groups, either transmembrane receptor-type PTPs (RPTPs) or cytoplasmic PTPs (2). RPTPs contain extracellular domains often resembling adhesion receptors, a single transmembrane domain, and either single or tandem catalytic domains in the intracellular sequence. Cytoplasmic PTPs consist of a single catalytic domain with various amino- or carboxy- terminal protein-binding motifs such as SH2 or FERM domains. The diversity in structure of the non-catalytic domains of PTPs determines their classification into subgroups and can affect variations in binding partners, localization and function.

1. Cell-Matrix adhesion

PTPs and adhesion to extracellular matrix

Transmembrane receptors of the integrin family are responsible for adhesion to many different ECM proteins, including fibronectin, laminin and collagen (3). For cells in tissue culture, sites of strong adhesion to the ECM are known as focal adhesions and they serve to anchor bundles of microfilaments (stress fibers) to the plasma membrane via integrins. Focal adhesions not only play a structural role, but also act as scaffolds for numerous signaling pathways downstream from integrin-mediated adhesion. Prominent among these signals is tyrosine phosphorylation of proteins at the cytoplasmic face of focal adhesions catalyzed by PTKs such as FAK and Src family kinases (SFKs) (4). Engagement of integrins in itself is

insufficient to induce the tyrosine phosphorylation and activation of FAK; integrin clustering at focal adhesions is required (5). The aggregation of integrins and resulting tyrosine phosphorylation at these sites is driven by myosin dependent cytoskeletal forces. This is, in turn, stimulated by the RhoA/Rho kinase pathway or pathways activating myosin light chain kinase (6). During integrin-induced adhesion, in parallel with the activation of FAK and SFKs, there is a general inhibition of PTP activity (7).

In comparison with the large amount known about the role of PTKs in focal adhesions, much less is known about PTPs. Several studies have reported changes in tyrosine phosphorylation within focal adhesions in response to manipulating specific PTPs. However, the identification of PTP targets within focal adhesions has been difficult and sometimes contradictory. The problem arises because tyrosine phosphorylation is important not only as a consequence of integrin-mediated adhesion, but it is also involved in many upstream signaling pathways that affect integrin clustering and focal adhesion assembly. Consequently, it is often difficult to discern whether manipulation of a PTP is directly affecting the phosphorylation of a protein downstream from integrins, or whether it is affecting focal adhesion assembly or disassembly, and thereby indirectly affecting tyrosine phosphorylation of focal adhesion components. Strategies used for investigating the roles of specific PTPs have included over-expression of wildtype PTPs, expression of mutant or catalytically dead PTPs and elimination of specific PTPs, either by knockout or siRNA strategies. However, all of these approaches may influence upstream signaling, and can be misleading when the readout is the tyrosine phosphorylation of focal adhesion components. One of the best approaches to identify specific targets is to use catalytically dead PTPs to "trap" their substrates (8).

PTPs can affect integrin-mediated adhesion and the tyrosine phosphorylation that occurs in focal adhesions by acting at least at three different levels. They can affect signaling upstream, for example, by regulating the activities of GEFs and GAPs for Rho proteins; they can act proximal to integrin engagement, for example by regulating Src kinase activity; or they can dephosphorylate downstream targets, some of which may feedback to influence upstream signaling pathways affecting focal adhesion assembly and turnover.

Upstream regulation of Rho protein activity by protein tyrosine phosphatases: PTP-PEST, Shp-2 and LMW-PTP

Important upstream regulators of cell matrix adhesions are members of the Rho family of small GTPases (9). In humans, this family of regulatory proteins includes approximately 20 proteins, although most work has been focused on three ubiquitously expressed members, RhoA, Rac1 and Cdc42. Like other G proteins, these proteins are active in the GTP-bound form and are inactive when GTP is hydrolyzed to GDP. Activation of Rho proteins is catalyzed by guanine nucleotide exchange factors (GEFs) which stimulate exchange of bound GDP with GTP from the cytoplasmic pool. Most Rho proteins have intrinsic GTPase activity, which is further stimulated by GTPase activating proteins (GAPs). Many GEFs and GAPs are regulated by tyrosine phosphorylation. Consequently, PTPs can profoundly influence the cycle of Rho protein activation by regulating the state of phosphorylation of GEFs and GAPs (Figure 1.1). Examples of PTPs that have been reported to regulate Rho protein activity are given in Table 1.

PTP-PEST is one PTP that affects adhesion and migration, in part by regulating the activity of Rho proteins. Over-expression of PTP-PEST depresses membrane ruffling at the

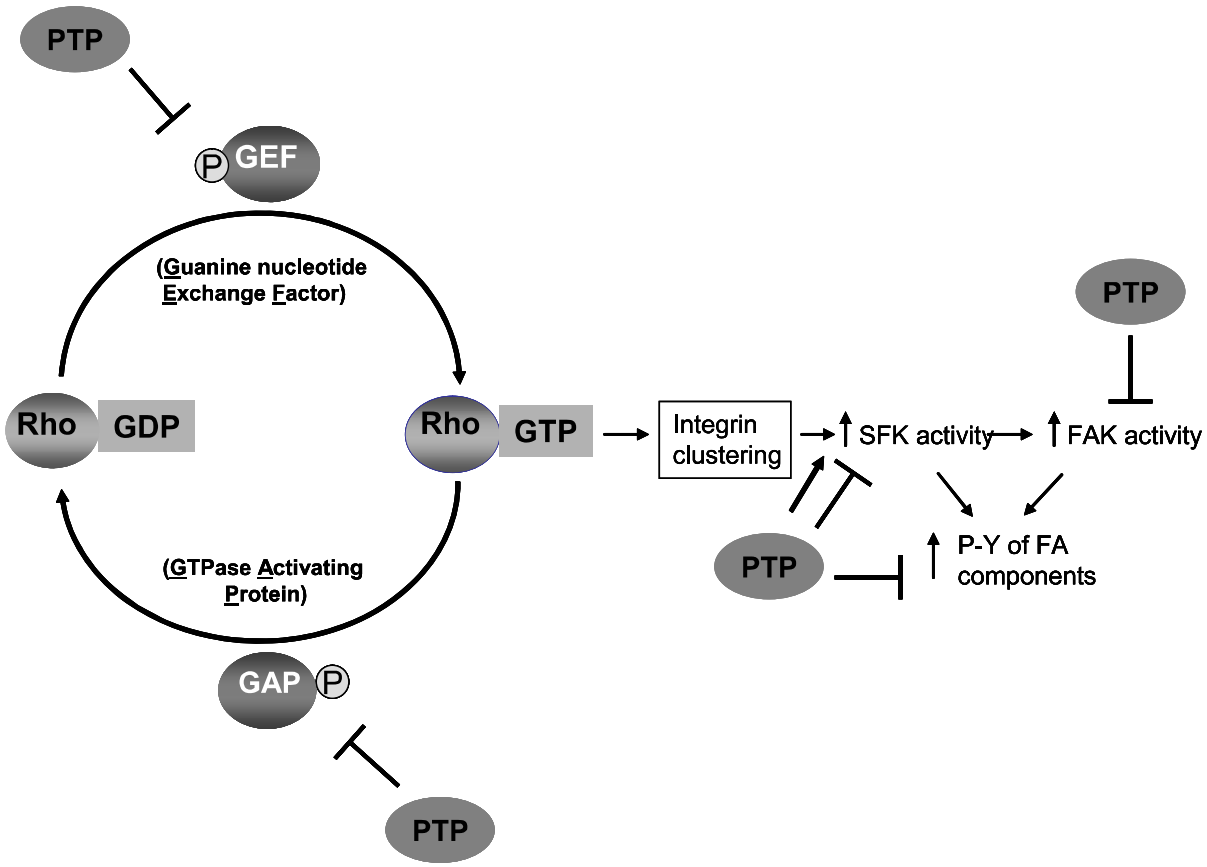


Figure 1.1. PTP regulation of integrin-mediated adhesion signaling and focal adhesions. Upstream, the clustering of integrins is determined by RhoA-GTP levels (activity). PTPs can regulate the activity of Rho proteins by controlling the phosphorylation states of GEFs and GAPs. Downstream, integrin clustering leads to SFK and FAK activation. PTPs can both activate and inhibit SFKs by removing inhibitory or activating phosphorylations. Regulating the tyrosine phosphorylation of downstream targets such as FAK regulates the dynamics and disassembly of focal adhesions.

Table 1. Regulation of Rho-GTPase activity by PTPs

PTP	GTP LEVEL	TARGET	CITATION
Shp-2	↑ RhoA-GTP ↑RhoA-GTP ↓RhoA-GTP	p190RhoGAP Unknown Vav, Vav2	(12) (13*, 14) (15, 16*)
PTP-PEST	↓Rac1-GTP	Unknown GEF	(10)
LMW-PTP	↑RhoA-GTP	p190RhoGAP	(17)

* implied from phenotype, GTP levels not measured

leading edge of cells due to decreased Rac1 activity (10). Conversely, in PTP-PEST-/- cells Rac1 activity is elevated and sustained in cells plated on fibronectin (11).

Several pathways by which PTP-PEST might regulate Rac activity are suggested from published studies. PTP-PEST has been shown to bind and act on both p130Cas and paxillin (18, 19) (Table 2). Both of these proteins, when tyrosine phosphorylated, interact with Rac GEFs. Tyrosine phosphorylated p130Cas and paxillin bind the adapter Crk (20-22), which in turn recruits the Rac GEF DOCK180 (23, 24). In addition, paxillin binds the Rac GEF PIX via the adapter protein Pkl/Git (25). Therefore, a loss of PTP-PEST may increase Rac activity by increasing the pool of phosphorylated p130cas and paxillin, thus preserving their interactions with and regulation of their GEF binding partners. Recent work has revealed that trapping mutants of PTP-PEST also bind Vav2 (11), a ubiquitously expressed GEF regulated by tyrosine phosphorylation (26). This observation raises the possibility that PTP-PEST may directly regulate Rac activity by controlling the phosphorylation state of this GEF without the need for adaptor proteins such as p130cas/paxillin.

The increased Rac1 activity found in PTP-PEST-/- fibroblasts would be predicted to increase migration, but these cells actually have reduced migration rates (27). Examination of cell morphology reveals prominent ruffling membranes/lamellipodia (hallmarks of active Rac1), but the cells develop elongated tails, indicative of problems in detaching from the substrate (11). Elongated tails are often associated with low RhoA activity (28). Through interactions with both a Rac GEF and an as yet unidentified Rho GAP, PTP-PEST may regulate the activities of both GTPases, thereby influencing migration by controlling membrane ruffling and tail retraction. However, an inability to disassemble focal adhesions

Table 2. Binding partners and substrates for PTPs involved in regulating cell-matrix adhesion

PTP	BINDING PARTNERS	SUBSTRATES
SHP-2	SHPS-1 (29, 30)	FAK (31) SHPS-1 (29, 30) PAG (32) Src (30) Vav2 (16) p190RhoGAP (12)
PTPα	Src-family kinases (33) $\alpha_v\beta_3$ integrins (34)	Src (35, 36) Fyn (35) Yes (33)
PTP1B	p130cas (37) β_1 integrins (38)	P130cas (37) Src (39)
PTP-PEST	P130cas (19) Paxillin (18) csk (40)	P130cas (19) Paxillin (18) PYK2/CAKβ (41) Vav2 (10)

in the rear would also account for the phenotype of the PTP-PEST null cells. Focal adhesion disassembly is regulated by tyrosine phosphorylation as well and will be discussed below. These results point to the complexity of phenotypes generated by PTP knockouts due to the actions of PTPs on multiple targets.

Another PTP implicated in regulating Rho protein activity is Shp-2. Here, conflicting results have been obtained, with some groups reporting that Shp-2 inhibits RhoA activity (15, 16), while other groups find that Shp-2 stimulates RhoA activity (13, 14), and still others suggest that Shp-2 can exert both positive and negative regulatory effects on RhoA activity (42). One target for Shp-2 is p190RhoGAP, a widely expressed GAP for RhoA (43) (Table 2). The activity of p190RhoGAP is stimulated by tyrosine phosphorylation (12, 43). By dephosphorylating p190RhoGAP and so suppressing its GAP activity, Shp-2 can elevate RhoA GTP levels (i.e. activate RhoA). Paradoxically, Shp-2 is one of the PTPs that stimulates Src activity (see below), and Src is responsible for phosphorylation and activation of p190RhoGAP (44). Consequently, Shp-2 can act on both sides of the phosphorylation equation regulating p190RhoGAP activity. In addition, Shp-2 may be one of the PTPs that inactivates Rho GEFs that are regulated by tyrosine phosphorylation (15, 16). Thus, the apparently conflicting role of Shp-2 with regards to RhoA activity could be reconciled by the differential action of the PTP on targets that can either positively or negatively regulate RhoA activity.

One non-classical PTP that warrants mentioning in the context of RhoA activity is LMW-PTP. It has been reported to act downstream of Src to regulate the phosphorylation state of p190RhoGAP, thereby controlling Rho-mediated cytoskeletal rearrangement (45). LMW-PTP has also been implicated in the crosstalk between Rac1 and RhoA, in which Rac1

mediated generation of reactive oxygen species was observed to inhibit LMW-PTP. This elevated p190RhoGAP tyrosine phosphorylation and activity, suppressing RhoA activity (17).

PTPs acting proximal to integrins: Shp-2, PTPα, and PTP1B

What is the initiating signal downstream from integrin-mediated adhesion? Several studies have implicated Src family kinases (SFKs) in some of the earliest steps downstream from integrins and preceding the activation of FAK (44, 46, 47). SFKs have been shown to bind β-integrin cytoplasmic domains (48, 49), which has prompted investigation into how these kinases are regulated in response to interaction of the integrins with their ligands. SFKs are held in an inhibitory state by two intramolecular interactions. One interaction involves the SH3 domain binding to the linker region between the kinase and SH2 domains. This constraint may be removed by association of the SFK with integrin cytoplasmic domains (39). The second intramolecular constraint to SFK activity involves binding of the SH2 domain to the phosphorylated C-terminal tyrosine residue (Y527 in avian, Y529 in mammalian cells). This inhibitory phosphorylation of Src is catalyzed by C-terminal Src kinase (Csk). Csk complexes with Src and inactive integrin αIIb/β3 (49). A PTP must dephosphorylate the C-terminal tyrosine in order for SFK activation. In addition, for full activity, phosphorylation of Y416 must occur in the activation loop within the kinase domain. Activation by removal of the C-terminal phosphate raises the possibility that PTPs may be involved in the initiation of the signaling downstream from integrin engagement or clustering. Several PTPs are capable of activating SFKs and, in the context of integrins, three have been studied, Shp-2, PTPα, and PTP1B (30, 31, 33, 36, 39).

Cells expressing a truncated form of Shp-2 (lacking the N-terminal SH2 domain), reveal diminished activation of Src and elevated phosphorylation of the inhibitory site, Y529, in response to adhesion to ECM (30, 32). These cells spread more slowly and display reduced tyrosine phosphorylation of FAK, paxillin and p130Cas (30, 31). In addition, Shp-2 may indirectly regulate Src activity by regulating the recruitment of Csk to the membrane. Csk is recruited to the membrane via association with tyrosine phosphorylated PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains), a transmembrane glycoprotein. Shp-2 dephosphorylates PAG and abolishes the Csk binding site, resulting in a reduction in membrane associated Csk and a reduction of Csk-mediated Src inhibition (32). Thus Shp-2 may activate Src both by directly acting on its C-terminal phosphorylation site and by inhibiting Csk recruitment. A protein that may act in parallel to PAG is SHPS-1 (SIRPα1). Like PAG, SHPS-1 recruits Shp-2 to the membrane and is a target for its activity (29, 30).

PTPα is a receptor type, transmembrane PTP involved in the activation of SFKs and in integrin signaling pathways. Ectopic expression of PTPα enhances the dephosphorylation of the c-terminal Y529, strongly activating src and fyn kinases (36). Cells lacking PTPα spread more slowly and contain decreased tyrosine phosphorylation of focal adhesion components (35, 50). The decrease in tyrosine phosphorylation of FAK, especially at autophosphorylation site Y397, in PTPα-/- cells suggests that this phosphatase lies between integrins and the activation of FAK (51). PTPα and the integrin αvβ3 co-immunoprecipitate from cells spreading on ECM substrates (34). This association has been shown to be involved in the activation of SFKs following integrin engagement, which, in turn, is involved in the reinforcement of integrin-cytoskeletal forces in response to tension (34). In this study,

the SFK involved was Fyn rather than Src. No evidence for an interaction between β1 integrins and PTPα was seen, but because similar downstream responses are observed for β1 and β3 integrins, it seems likely that parallel pathways may operate, possibly involving different PTPs and different SFKs.

In platelets, activation of Src occurs rapidly in response to integrin engagement, whereas FAK activation is a relatively late event (46). The association of Src with the β3 cytoplasmic domain involves binding via its SH3 domain, relieving one of the inhibitory constraints on Src (39). With platelet αIIb/β3, the activation involves release of associated Csk from the integrin/SFK complex and the subsequent recruitment of PTP1B. Interestingly, the recruitment of PTP1B requires tyrosine phosphorylation of PTP1B and is blocked by Src inhibitors (39). This implies that some level of Src activation must precede the recruitment of PTP1B. Shattil and colleagues propose a model in which binding of αIIb/β3 to fibrinogen induces micro-clustering of αIIb/β3, juxtaposing Src molecules so that these cross-phosphorylate on Y416. It is suggested that this results in initial activation sufficient to phosphorylate and recruit PTP1B, which, by removing the C-terminal phosphorylation of Src results in full activity. In this model, many of the subsequent tyrosine phosphorylations, including FAK activation, are triggered downstream from these initial events (39).

Differential PTP1B signaling in various cell types

With regard to its role in ECM adhesion, conflicting results have been reported for PTP1B. The finding that PTP1B binds and acts on p130Cas (37) led to experiments investigating the effects of expressing wildtype or mutant forms of PTP1B unable to bind to p130Cas in cell adhesion situations. Expression of wildtype but not mutant PTP1B slowed

fibroblast spreading and depressed tyrosine phosphorylation of p130Cas and other proteins in response to adhesion (52). In addition, the expression of wildtype PTP1B enhanced the assembly of focal adhesions with short thick stress fibers, and decreased cell migration. Consistent with these findings, depressing PTP1B expression in vascular smooth muscle enhanced tyrosine phosphorylation of p130Cas and stimulated migration (53). Seemingly contradictory results, however, were obtained in another study in which wildtype or catalytically dead PTP1B were expressed in L cells (38). In these experiments, expression of the wildtype PTP1B did not depress tyrosine phosphorylation in response to adhesion to fibronectin, whereas expression of a catalytically dead mutant did. Expression of the inactive mutant also suppressed Src activity and depressed cell attachment to a fibronectin substratum. Additionally, the cells expressing the mutant PTP1B displayed an elongated morphology, with focal adhesions that were reduced in size and number. A third study examined the behavior of fibroblasts derived from PTP1B null mice (54). In this work, the cells lacking PTP1B exhibited delayed spreading on a fibronectin-coated surface, but surprisingly little effect was found in terms of tyrosine phosphorylation in response to adhesion to fibronectin. However, when wildtype and PTP1B null cells were transformed with SV40Tag, effects on tyrosine phosphorylation were seen. Now the transformed null cells exhibited decreased tyrosine phosphorylation of p130Cas relative to transformed wildtype cells following short periods (20 min) of adhesion to fibronectin. These null cells also showed hyperphosphorylation of the inhibitory site in Src (Y527) in some situations. Notably, the SV40Tag-transformed fibroblasts revealed an increased expression of PTP1B relative to primary mouse embryo fibroblasts, possibly accounting for the differences between the transformed and primary cells.

Can these apparently conflicting observations with PTP1B be reconciled? As the authors have suggested, cell type differences may be critical, especially given that SV40Tag-transformed cells elevate expression of PTP1B (54). Cell types may also diverge both as to where PTP1B is acting in adhesion signaling pathways and in the degree of compensation by other PTPs. For example, in some cell types PTP1B may have a major role regulating the C-terminal inhibitory phosphorylation site of SFKs, whereas in other cell types different PTPs (e.g. PTPα or Shp-2) may be more important. P130cas is a major substrate for Src and so in cells in which PTP1B is regulating Src activity, one would predict lower phosphorylation of p130Cas when PTP1B is absent or inactive (38, 54). However, in other cell types where different PTPs may be more critical in regulating SFK activity, the effect of depressing PTP1B activity would be predicted to be less significant in terms of p130Cas phosphorylation. Since PTP1B can also dephosphorylate p130Cas, it would not be surprising in these cells to observe that over-expression of wildtype PTP1B decreases the phosphorylation of this target protein (52). One of the striking observations from Chernoff's group is that the cells over-expressing PTP1B revealed enhanced focal adhesions and stress fibers (52). This is suggestive of increased RhoA activity and could arise because of dephosphorylation and inactivation of a regulatory protein such as p190RhoGAP. However, this phenotype could also result from defective focal adhesion disassembly (see below).

Focal adhesion disassembly: Downstream regulation by PTPs

With the discovery of FAK activation in response to integrin-mediated adhesion, it was widely interpreted that tyrosine phosphorylation promoted focal adhesion assembly. However, the phenotype of FAK knockout cells, as well as cells in which FAK was displaced

from focal adhesions, revealed robust and stable focal adhesions in the absence of FAK and tyrosine phosphorylation detectable by immunofluorescence (55, 56). Rather than assembly of focal adhesions, FAK activity correlated with focal adhesion turnover and disassembly (4). While several pathways downstream of FAK could contribute to focal adhesion disassembly (4, 25, 57-61), a novel, endocytic pathway (62) suggests a potentially important role for an as yet unidentified PTP. In this work, tyrosine phosphorylated FAK was found to recruit dynamin to focal adhesions (62). Dynamin is a protein involved in endocytosis and expression of a dominant negative form of dynamin that inhibits endocytosis blocked focal adhesion disassembly. The association of FAK with dynamin is mediated by the adaptor protein Grb2, which binds to phosphorylated Y925 in FAK and to the proline rich region of dynamin. Expression of the non-phosphorylatable FAK mutant, Y925F, failed to rescue focal adhesion disassembly in FAK null fibroblasts (62).

Identification of the PTP that removes the phosphate from Y925 in FAK will be important. Based on the above information, it would be predicted that this PTP would have a key role in regulating focal adhesion disassembly and, by extension, in regulating cell migration. In order for cells to migrate, focal adhesions must be disassembled so that strong adhesions to the underlying ECM can be released. The PTP that mediates dephosphorylation of Y925 in FAK would be anticipated to increase focal adhesion stability and decrease migration when it is over-expressed, but to increase migration rates and the turnover of focal adhesions when it is inhibited or knocked out. This phenotype matches that described by Liu and coworkers when they over-expressed PTP1B in fibroblasts (52) and it will be interesting to learn whether PTP1B or another PTP regulates the phosphorylation status of Y925 in FAK.

2. Cell-Cell adhesion

PTPs and cell-cell junctions

Epithelial tissues typically display stable cell-cell adhesion that is accompanied by prominent cell junctions between interacting cells. Tight junctions (TJs) are responsible for the barrier function of many epithelia, whereas adherens junctions (AJs) and desmosomes mediate strong intercellular adhesion. The assembly and function of TJs is typically dependent on the state of AJs, so that modulating AJ function often affects TJ barrier properties. The major adhesion molecules in both AJs and desmosomes belong to the cadherin family; however, cadherins can also contribute to adhesions between cells where distinct junctions do not develop and where adhesion is more dynamic. Additional adhesion molecules, such as nectins (63), are also present in AJs, but we will focus here on cadherins. Whereas the extracellular domain of cadherins participates in calcium-dependent homophilic adhesion, the cytoplasmic domain binds p120ctn and β-catenin (64-66). The former regulates the stability of cadherins on the cell surface (67), and β-catenin provides a link to α-catenin and the actin cytoskeleton, although the details of the bridge remain controversial (68). Tyrosine phosphorylation of cadherins and their associated proteins has major effects on the stability of adherens junctions (69, 70). In early work, it was shown that inhibiting PTPs with pervanadate elevated tyrosine phosphorylation in adherens junctions and promoted the disassembly of these structures (71). However, the same group later observed that in some situations elevation of tyrosine phosphorylation first transiently stimulated AJ assembly before resulting in the eventual disassembly of the same structures (72). Activation of PTKs or inhibition of PTPs can lead to increased tyrosine phosphorylation of members of the cadherin-catenin complex, dissociation of the AJ from the cytoskeleton and disruption of

cell-cell adhesion (73-75). For example, phosphorylation of tyrosine residues 755 and 756 on E-cadherin leads to its ubiquitination and subsequent endocytosis, resulting in loss of junctional integrity (76). Similarly, phosphorylation of Y658 and Y731 in the cytoplasmic domain of VE-cadherin prevents binding of p120ctn and β-catenin, respectively, and causes a decrease in barrier function (77). Therefore, maintenance of junctional integrity is regulated in part by reversible tyrosine phosphorylation that results from a competing balance of PTK and PTP activity. Receptor-PTPs such as PTPμ, DEP-1, and VE-PTP, as well as the cytosolic PTPs, PTP1B and Shp-2, have been shown to bind to members of the cadherin-catenin complex (78-83) (Table 3) and to regulate cell-cell adhesion by means of regulating phosphorylation of the cadherin-catenin complex.

Direct regulation of the cadherin-catenin complex

The extracellular domains of PTPμ can mediate cell-cell adhesion via homophilic interactions (84, 85). Together with its binding to p120ctn and the cytoplasmic domain of cadherins, the homophilic interactions of PTPμ combine to localize it to cell-cell junctions (78, 79, 86, 87). Localization at AJs orients PTPμ's catalytic domain in close proximity with substrates VE-cadherin and p120 catenin (79, 87). In addition, knockdown of PTPμ by siRNA increases permeability of endothelial monolayers demonstrating its role in the regulation of junctional integrity (87). Although many of the effects of PTPμ at AJs are undoubtedly due to its phosphatase activity, other domains of the protein may also contribute to junctional stability. Evidence for this came from a

study of prostate carcinoma cells lacking endogenous PTPμ which were unable to form AJs even though E-cadherin and the catenin proteins were present. Expression of PTPμ restored

cadherin-mediated cell-cell adhesion, but strikingly this could also be achieved by expression of catalytically dead PTPμ (88). This finding suggests that PTPμ can act as a scaffold and recruit additional regulatory proteins to sites of cell-cell adhesion.

Another PTP important for junction formation and maintenance is high cell density enhanced PTP-1 (DEP-1). Expression of DEP-1 is increased more than ten-fold in many cell types as they approach confluence, suggesting it contributes to cell-cell adhesion and contact inhibition of growth (89). DEP-1 is present at the apical surface of endothelial cells, but also co-localizes with VE-cadherin at intercellular junctions (90, 91). DEP-1 indirectly associates with VE-cadherin by binding to p120ctn, γ-catenin (plakoglobin) and β-catenin (Table 3); DEP-1 regulates their phosphorylation state, preserving their interactions with the cadherins, and promoting cell-cell adhesion (80, 90). The role of DEP-1 in organizing cell-cell junctions is supported by the observation that in cells lacking strong cell-cell adhesion, such as fibroblasts, overexpression of DEP-1 results in a change in localization of cadherins from discrete areas of cell-cell contact to large areas reminiscent of continuous AJ found in epithelial cells (92).

A PTP in the same family as DEP-1 is vascular endothelial PTP (VE-PTP), which is selectively expressed in endothelial cells, and binds to VE-cadherin but not to β-catenin (93). VE-PTP associates with VE-cadherin via its membrane-proximal extracellular domain and its recruitment results in the dephosphorylation of VE-cadherin (81). Expression of wild type VE-PTP decreases paracellular permeability in endothelial monolayers, while the catalytically inactive mutant has no effect, indicating that the phosphatase activity of VE-

Table 3. Binding partners and substrates for PTPs involved in the regulation of cell-cell junctions

PTP	BINDING PARTNERS	SUBSTRATES
PTPμ	E cadherin (78) PTPμ (84, 94) RACK-1 (95) p120ctn (79) VE-cadherin (87)	p120ctn (79) VE-cadherin (87)
PTPκ	PTPκ (96) β-catenin, plakoglobin (97)	β-catenin (97)
PTP-LAR	β-catenin (98, 99) plakoglobin (99)	β-catenin (99)
DEP-1	p120ctn (80) Matrigel component (100)	p120ctn, β-catenin, plakoglobin (80)
VE-PTP	VE-cadherin (81)	VE-cadherin (81)
SHP-2	β-catenin (82) PECAM (101, 102)	β-catenin (82)
PTP1B	N-Cadherin (83, 103, 104)	β-catenin (83, 103, 104)

PTP acting on VE-cadherin is necessary for maintaining the integrity of endothelial junctions (81).

Cytosolic PTPs such as PTP1B and Shp-2 have also been implicated in regulating cell-cell adhesion by controlling phosphorylation of cadherin/catenin proteins (82, 83, 103). PTP1B binds directly to the cytoplasmic domain of N-cadherin, an interaction that promotes β-catenin binding and targeting of the cadherin/catenin complex to the cell membrane (104). Expression of a catalytically inactive mutant of PTP1B disrupts cadherin-mediated adhesion, with concomitant increases in tyrosine phosphorylation of β-catenin and reduction in the association of N-cadherin with the actin cytoskeleton, suggesting that the catalytic activity of PTP1B is important for junctional maintenance (83, 103). Shp-2 is another cytosolic PTP associated with the cadherin/catenin complex in confluent endothelial cell monolayers, specifically interacting with β-catenin (82). Thrombin treatment of endothelial cells induces Shp-2 tyrosine phosphorylation and dissociation from β-catenin followed by junctional breakdown and the formation of large intercellular gaps, thus supporting the hypothesis that Shp-2 localization to junctions also helps maintain endothelial junctional strength and integrity (82). The loss of Shp-2 binding is accompanied by an increase in phosphorylation of β-catenin, plakoglobin and p120ctn. AJs are not the only adhesive complexes where PTPs play a role in regulating signal transduction. Shp-2 binds to platelet endothelial cell adhesion molecule-1 (PECAM-1) (101, 102) and intercellular adhesion molecule 1 (ICAM-1) (105, 106). In response to ICAM-1 engagement, phosphorylated ICAM-1 binds to Shp-2 and this interaction is necessary for Src activation as well as p38 MAPK activation (106).

The concentration of PTPs, both cytosolic and transmembrane, at AJs indicates the importance of maintaining low levels of tyrosine phosphorylation at these sites, except when

the junctions need to be remodeled or disassembled. In general, the suppression of bulk PTP activity by inhibitors or the suppression of individual PTPs by siRNA elevates the tyrosine phosphorylation of cadherins and their associated proteins. In turn, this results in destabilization of the junctions and diminished epithelial or endothelial barrier functions.

Rho GTPases and cell-cell adhesion

Just as with cell-matrix adhesions (107), there is bidirectional interplay between Rho family GTPases and cell-cell adhesions. Not only is the assembly of AJs affected by the activities of Rho proteins, but cell-cell adhesion can stimulate or depress the activities of these G proteins, suggesting complex feedback loops. Several studies have shown that Rac1 activation promotes assembly of epithelial AJs and inhibition of either Rac1 or RhoA activity results in disassembly (108-110). Conversely, other studies show that high levels of active Rac1 or RhoA can actually disrupt both TJs (111) and AJs (112, 113); this might reflect different cellular contexts or the need for tight regulation of GTPase activity. The formation of AJs can activate Rac1 and Cdc42 (114, 115) but inhibit RhoA (115). PTPs may play critical roles in these upstream and downstream pathways by regulating the phosphorylation and activity of GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (107). For example, p190RhoGAP tyrosine phosphorylation was implicated in the depression of RhoA activity downstream from cadherin engagement (116).

With endothelial monolayers, several agents that promote increased permeability also stimulate Rac1 activity. PTPs are also likely to be important in this response to Rac1 because in these cells Rac1 has been shown to generate reactive oxygen species (ROS) (117). In turn, ROS are potent inhibitors of PTPs (118) and are responsible for the elevation in junctional protein tyrosine phosphorylation that results from Rac1 activation (113, 117, 119).

Regulation of PTP activity

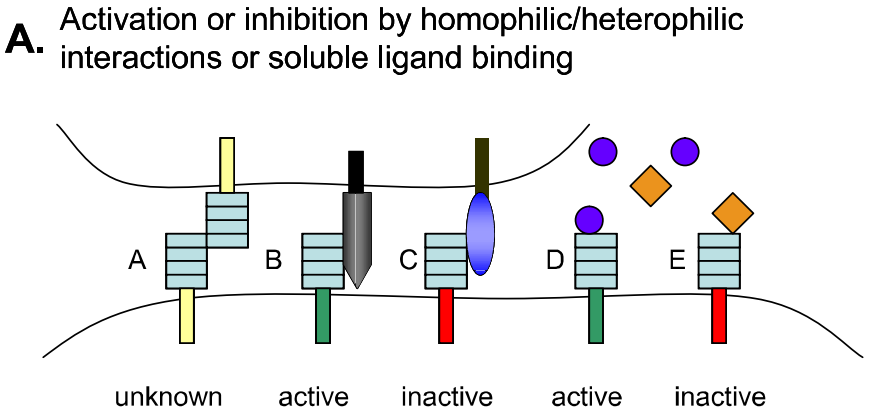
The resemblance of the extracellular domains of RPTPs to cell adhesion molecules has stimulated a search for potential binding partners and by extension, for modes of regulation via such interactions. Several mechanisms for modulation of specific PTP activity exist (Figure 1.2). In some cases, homophilic interactions have been detected, as first shown with PTPμ (84, 85). Two closely related PTPs, PTPκ and PTPλ, have similarly been shown to interact in a homophilic manner (96, 120). The extracellular domains of several RPTPs participate in heterophilic interactions with ECM or other components. For example, LAR binds the laminin/nidogen complex, which is prominent in many basement membranes (121). Similarly, PTPσ binds to heparin sulfate proteoglycans (122) and PTPα binds contactin (123). Although several interactions involving the extracellular domains of RPTPs have been identified, in most cases no effect on the activity of the PTP has been demonstrated. One exception is the interaction of PTPβ/ξ with pleiotrophin, which inhibits PTP activity and results in an increase in β-catenin phosphorylation (124). Pleiotrophin is a heparin binding growth associated molecule and its interaction with PTPβ/ξ promotes cortical neuron migration (124). In

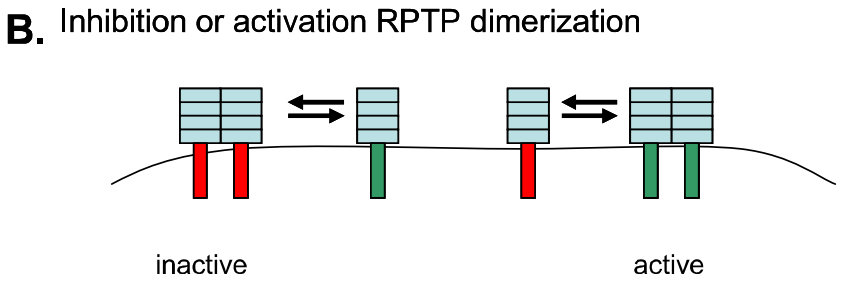
contrast, DEP-1 phosphatase activity is up-regulated by the binding of its extracellular domain to an unknown component of Matrigel® (100). The observation that the activities of RPTPs can be regulated by the interactions of their extracellular domains will be broadly important if further work can establish that this is a general characteristic of RPTPs. It is easy to imagine many scenarios in which tyrosine phosphorylation levels could be regulated

by interactions of the extracellular domains of RPTPs with other cells, with matrix molecules or soluble ligands (Figure 1.2A).

Dimerization of RPTPs provides another potential mode of regulating their activity. RPTPs have traditionally been considered to be inactive when dimerized and active when monomers (125). This idea was first proposed when a chimera of the extracellular domain of EGFR fused to the intracellular domain of CD45 was inhibited by EGF-induced dimerization (126). Further support was generated when studies revealed RPTPα exists as dimers on the cell surface, and that this dimerization inhibits the activity of the phosphatase via an interaction of the tandem catalytic domains, preventing the binding of substrates (127-129). This suggests a model where RPTPs exist as inactive dimers on the cell surface in the absence of a ligand and binding of the ligand dissociates the dimers and activates the PTP (Figure 1.2B). However, dimerization-induced inactivation of RPTPs through blockage of the active site by an opposing PTP domain may not be a universal mechanism. Structural analysis of the membrane-proximal catalytic domain of PTPμ and the entire cytoplasmic domain of PTP-LAR suggests that these PTPs are not inhibited by dimerization (130, 131). In addition, RPTPs such as DEP-1 and VE-PTP contain a single catalytic domain and therefore may also not be inhibited by dimerization.

The regulation of PTP activity by ROS is an area developing very rapidly and too large to review in detail here. The cysteine residue in the catalytic site of classical PTPs has long been known to be sensitive to oxidation and this has been the basis for broad specificity inhibitors, such as H_20_2 and pervanadate. Subsequent work has shown that reversible oxidation of the catalytic cysteine can provide a physiological mechanism for regulating PTPs (118) (Figure 1.2C). The generation of ROS was originally identified as a defense





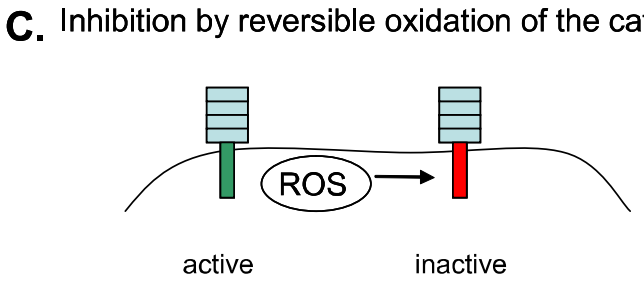


Figure 1.2. Modes of regulation of receptor protein tyrosine phosphatase activity. (a) RPTPs bind soluble ligands (D,E) or interact in *trans* with proteins on adjacent cells by either a homophilic (A) or heterophilic (B,C) mechanism. In some instances, such as homophilic interaction, it is not clear whether the interaction affects activity (shown with a yellow cytoplasmic domain). However, heterophilic interactions can be either activating (shown in green) or inhibitory (shown in red) and appear to be ligand/PTP specific. (b) RPTPs also interact in *cis* on the cell surface. In most cases dimerization is associated with inactive PTPs. However, the known conformational change by which dimerization inhibits activity is not consistent for the interactions of all RPTPs. Therefore, in some cases dimerization may increase PTP activity. Further investigation into this is needed. (c) In several cell types reactive oxygen species (ROS) are generated in signaling pathways, such as downstream from Rac1 activation. The reversible oxidation of the catalytic cysteine by ROS inhibits PTP activity.

mechanism in the phagocytic killing of bacteria by leukocytes, but more recently the generation of low levels of ROS by other cells has been recognized as a widespread occurrence with important physiological consequences (132). The formation of ROS is downstream of active Rac1 (17, 117). For example, in endothelial cells, active Rac1 promotes endothelial permeability and is associated with increased tyrosine phosphorylation of junctional proteins, such as VE-cadherin and β-catenin (117, 119). These effects are blocked by inhibiting the generation of ROS and are mimicked by pervanadate (117). Whether these effects are the result of one or a few PTPs being inhibited or reflect broad inhibition of all PTPs within these cells has not been established. It will be interesting to determine the degree to which ROS can act locally on one or a few PTPs rather than globally by inhibiting all PTPs within a cell. Techniques have been developed for analyzing the reversible oxidation of PTPs (133) and so this area should advance rapidly. There are many physiological situations in which Rac1 is activated and it will be important to determine whether the generation of ROS and the consequent inactivation of PTPs is a general signaling pathway downstream from Rac1 or whether this only occurs in particular situations.

DEP-1 (density enhanced protein tyrosine phosphatase-1)

As mentioned above, there are both cytoplasmic and receptor protein tyrosine phosphatases. This thesis focuses on the role of RPTPs in general and DEP-1 in particular, in regulating cell-cell adhesion and signaling events. Also known as PTP-η, PTPRJ, and CD148, DEP-1 is a single pass transmembrane protein with an extracellular domain

consisting of 8 fibronectin type III repeats, a transmembrane domain and a single intracellular catalytic domain (89, 134). Classification of RPTPs is based on the sequence motifs of their extracellular domains (Fig. 1.3) and these characteristics classify DEP-1 as a type III RPTP (135). Several reviews discuss the structural differences of all PTPs in more detail (2, 136-138). DEP-1 protein levels were enhanced greater than 10 fold in fibroblasts as the cells were grown to high density, which led to its name density enhanced protein tyrosine phosphatase-1 and to the hypothesis that DEP-1 may be involved in mechanisms of cell differentiation and contact inhibition of growth (89). This hypothesis has been supported by several studies in which increased expression of DEP-1 inhibited the growth of several cancer cells *in vitro* (139-142). Originally DEP-1 was cloned out of a human cDNA library but homologues have been identified in rat and mouse (134, 143, 144) and DEP-1 is considered to be ubiquitously expressed (143).

In the last decade the development of the substrate trapping mutant has helped to identify potential substrates of PTPs. These trapping mutants are point mutations of key residues surrounding the active site important for enzyme-substrate recognition and catalytic activity (145-147). The conserved cysteine in the catalytic pocket responsible for the phosphor-enzyme interaction is mutated to a serine (C/S) creating a catalytically dead mutant. This mutant retains some ability to bind to substrates but has most extensively been used as a catalytically dead mutant in studies examining phosphorylation of putative substrates. In addition, the conserved aspartic acid in the WPD loop of the catalytic domain can be mutated to an alanine (D/A). This mutation prevents the aspartic acid from acting as a general acid and facilitating the protonation of the oxygen atom in the tyrosyl leaving group and traps the substrate within the phosphatase's catalytic domain (8). This mutation allows

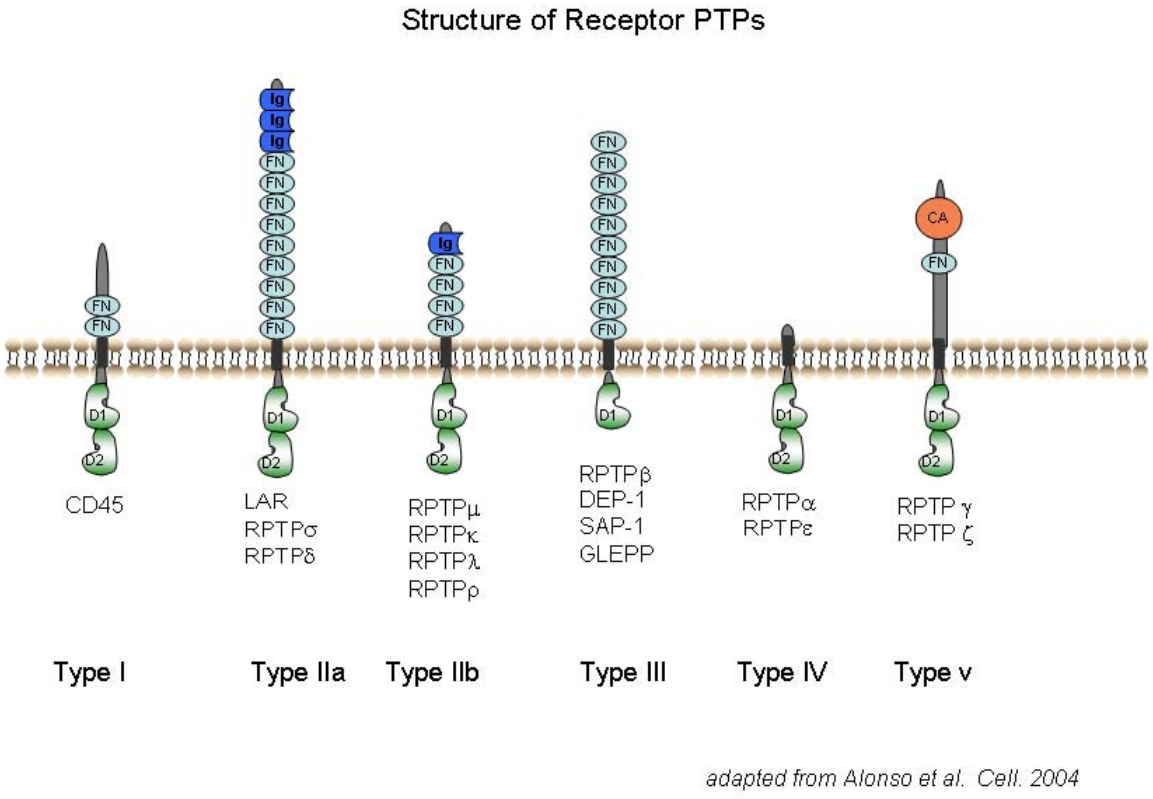


Figure 1.3. Structure of receptor protein tyrosine phosphatases**.** RPTPs are divided into subcategories based on the structure of their extracellular domain. Shown here the 5 most common types. Blue ovals (FN), fibronectin type III repeats; dark blue Ig, Ig like domains; orange circle (CA), carbonyic acid; D1, first catalytic domain, often active, D2, second catalytic domain, often inactive, dark gray, transmembrane domain. Adapted from (2).

for the binding of the phosphorylated substrate but prevent its removal, turning a transient interaction into a

stable one. Several substrates for DEP-1 have been identified using this trapping mutant, including p120 catenin, β-catenin, the p85 subunit of PI3K, and the HGF receptor Met (80, 148, 149). Physiological effects of these interactions still need to be elucidated, however, there is a great body of literature demonstrating DEP-1's regulation of and involvement in several signaling pathways in different cell types and these will be reviewed in the following sections.

Role of the extracellular domain of DEP-1

The extracellular domain of DEP-1 is highly N-glycoslyated and believed to be acting as an adhesion receptor involved in outside-in signaling (89, 134). The intrinsic PTPase activity of DEP-1 may be regulated or modulated by interacting with other cell surface molecules (ligand mediated signal transduction) as well as through dimerization of DEP-1 extracellular domains in cis. When cells or recombinant DEP-1 protein were incubated with Matrigel®, a preparation of extracellular matrix proteins, DEP-1 catalytic activity increased (100). The exact ligand that causes the increased catalytic activity is not known. This was the first evidence of increased catalytic activity of a RPTP by an extracellular ligand. A few years later, a research group developed an antibody to the extracellular domain of DEP-1 and tested its effect on endothelial cell growth (150). They discovered that antibody-induced ectodomain oligomeriztion of DEP-1 inhibited cell growth by increasing the catalytic activity of the cytoplasmic domain (150). This is particularly interesting due to the fact that other PTPs are believed to be inactivated when dimerized (126-129). Therefore, dimerization of

DEP-1 or binding to extracellular proteins can increase PTP activity. One proposed reason for the difference between other PTPs and DEP-1 is that DEP-1 has a single catalytic domain, not a tandem set of domains, and is not prone to the inhibitory binding of opposing PTP domains.

It is also possible that the extracellular domain of DEP-1 is important independent of its ability to signal to its catalytic domain. Mice with a knock-in mutation of DEP-1, where GFP replaced the intracellular catalytic domain, resulted in a dramatic embryonic lethal phenotype in homozygous mutants with mice dying before embryonic day 11.5 (91). These mice had disorganized vascular structures and cardiac defects (91). Interestingly, when the entire gene coding for DEP-1, PTPRJ, was deleted from mice they were viable, fertile and showed no gross anatomical alterations, suggesting PTPRJ is dispensable for normal growth and development of mice (151). Therefore, loss of the protein had no effect but the presence of the extracellular domain (without PTP activity) is lethal. This suggests that the extracellular domain of PTPRJ might act as functional ligand which is able to block pathways responsible for endothelial cell assembly necessary for correct vascularization (151).

DEP-1 regulation of growth factor receptors

A number of studies have examined DEP-1's role in modulating growth factor signaling by directly acting on the growth factor receptors. Knockdown of endogenous protein or expression of the catalytically dead mutant of DEP-1 in endothelial cells increased phosphorylation of VEGFR- 2 as well as p44/42 MAPK in response to VEGF, suggesting that DEP-1 negatively regulates VEGFR-2 activity (152). Specific residues

dephosphorylated by DEP-1 have not yet been fully investigated for this RTK. Biochemical studies both in vivo and using peptide substrates revealed that DEP-1 is able to directly dephosphorylate PDGF β-receptor in a site-selective manner (153). Interestingly, the regulatory Tyr 857 was not found to be a preferred site for DEP-1 dephosphorylation, instead Tyr 1021 showed the highest affinity for dephosphorylation (153). Biological responses triggered by PDGF β-receptor and other PTKs is determined by the SH2 domain containing proteins that associate with receptor in a phosphorylation site selective manner (154, 155). Therefore, DEP-1 may not be attenuating the RTK signal completely by acting on the regulatory tyrosine but modulating the signaling output by dephosphorylation of a subset of SH2-domain binding tyrosines (153). Similarly, HGF receptor kinase Met is also a substrate for DEP-1 with non-preferred targets in the tyrosines of the activation loop. Instead DEP-1 dephosphorylates C-terminal tyrosines known to be important for morphogeneis and Gab1 binding (149). Again, this result supports the role of DEP-1 in controlling the specificity of signals induced by the RTK rather than a simple "off switch". For these reasons, regulated activation and expression of PTPs is a possible mechanism for altering biological response following ligand stimulation of RTKs. Interestingly, LMW-PTP (a structurally distinct PTP from DEP-1) is also able to dephosphorylate PDGF β-receptor but with the reverse preference for tyrosine residues (156). This suggests that the preference for phospho-peptides of PDGF β-receptor by DEP-1 is not shared by other PTPs involved in PDGF β-receptor dephosphorylation and can provide an explanation for why several PTPs are able to act on the same substrate.

Several studies have also investigated the physiological effects of DEP-1 on PDGF signaling through overexpression and knockdown studies. DEP-1 expression in fibroblasts

inhibited PDGF-stimulated cell migration (92, 157), whereas DEP-1 -/- MEFs showed increased chemotaxis towards PDGF (158). Several PDGF-stimulated signaling events were negatively modulated by DEP-1 expression, with the most prominent effects being on phospholipase Cγ1, Ras and ERK1/2 activation (157). Another study also showed that the Ras-MAPK pathway as well as the PI3-kinase-Akt pathway was negatively regulated by DEP-1 protein levels (92). Although these studies are in agreement with regards to signaling effects and migration, contradictory results have been found for the effect of DEP-1 on cell-matrix adhesion. Jandt et al. found that DEP-1 expression was a positive regulator of cell adhesion, with DEP-1 expressing cells initially adhering faster but eventually reaching the same number as control cells over a 4 hr period (157). However, Kellie et al. found DEP-1 fibroblasts displayed a reduced number of cells both adhered and spread compared to control cells (92). This study supported its claim with reduced FAK phosphorylation compared to vector controls and reduced FA formation, both associated with decreased adhesion. Both studies were conducted in NIH3T3 cells and therefore the difference could lie in the matrix that was used (collagen 1 vs fibronectin) or the extent of over-expression of DEP-1. These studies enhance the receptor dephosphorylation data, demonstrating that cell adhesion and migration downstream of PDGF are affected by DEP-1 protein levels.

DEP-1 in cancer

In the last several years, evidence has been published linking DEP-1 with certain cancers. The chromosomal location of DEP-1 has been mapped to chromosome 11p11.2 on the short arm of chromosome 11 (134). Previously, loss of heterozygosity or deletion of sequences in the short arm of human chromosome 11 had been detected in various tumors of epithelial

origin, including breast cancer, bladder cancer, and hepatocellular carcinoma (159-161). Recent studies have looked more specifically at this chromosome, investigating the gene responsible for DEP-1 expression, PTPRJ. It has been found that human colon, lung, breast and thyroid cancers frequently have somatic missense mutations, loss of heterozygosity, or deletions of the PTRRJ gene (DEP-1) (162, 163). In addition, the mouse homologue gene, Ptprj, is a candidate for the mouse colon cancer susceptibility locus Scc1 (163). DEP-1 expression has been shown to be drastically reduced in multiple cancer cell lines and human cancers including thyroid, breast, pancreatic and colon cancers. (139-142, 164). Therefore, DEP-1 has been proposed to be a tumor suppressor. One way in which it may be acting as a tumor suppressor is by inhibiting cell proliferation. Studies have re-expressed DEP-1 in cancer cell lines lacking endogenous DEP-1 and found that there was a profound inhibition in cell proliferation (139-142, 164). In addition, endogenous DEP-1 protein levels have been reduced with shRNA constructs resulting in enhanced proliferation (164), supporting a inhibitory role for DEP-1 in regulation of cell growth. In fact, injection of adenovirus expressing DEP-1 into mice with tumors derived from injected pancreatic cancer cells blocked the growth of the tumors compared to untreated or control adenovirus treatment (142). Restoring DEP-1 expression could be a tool for gene therapy in human (pancreatic) cancers.

A number of mutations in the DEP-1 gene, PTPRJ, have been identified and create coding polymorphisms often found in human cancers. Gln276Pro, Arg326Gln, and Asp827Glu are mutations in the extracellular domain, specifically in the 2^{nd} and 8^{th} fibronection repeats (Fig. 1.4) (142, 163). Sequence alignments, secondary structure prediction and homology modeling have predicted with high confidence that most amino acid

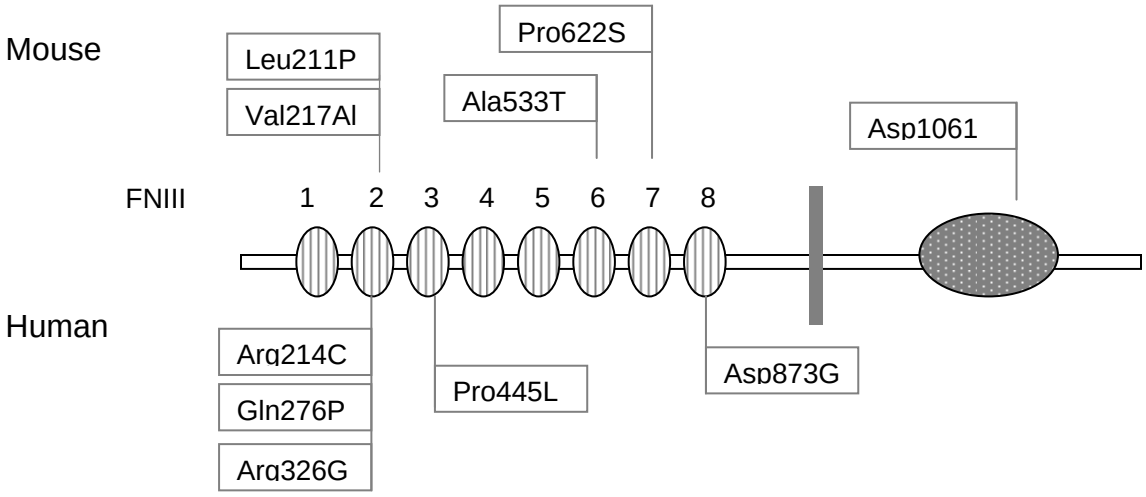


Figure 1.4. Polymorphisms of mouse and human PTPRJ. Schematic representation of polymorphisms that result in amino acid substitutions in both mouse and human. For mice, sequences from Balb/c and STS were compared and positions are from Balb/c sequence. For Human, comparisons were made from PTPRJ gene from colon cancer patients. Prediction of effects of substitution for Human only: The Arg214Cys and Arg326Gln substitutions are predicted to lose positive charges, Gln276Pro results in torsional stress, Pro445Leu releases torsional stress, and the effect of Asp873Glu has not been predicted. The Asp1061Glu substitution is not expected to affect the activity of the catalytic domain, because it is conservative (involving two acidic residues) and affects a residue relatively distant from the active site. FNIII is fibronectin type 3 domain (striped), the transmembrane domain is gray and the catalytic domain is the spotted oval. Modified from Ruivemkamp 2002 (163).

substitution in the extracellular domain of PTPRJ and Ptprj (the mouse homologue) occur in exposed regions available for interactions with ligands and other proteins and could affect the signaling process (163). For example, the Gln276Pro mutation results in torsional stress and loss of available hydrogen bond-forming group, eventually leading to conformational changes in other residues in the structural "neighborhood" and potentially altering the general properties for interaction with extracellular ligands (163). The Gln276Pro polymorphism also seems to play a critical role in susceptibility to certain human neoplasias (162). In fact, PTPRJ genotypes homozygous for Gln276Pro and Arg326Gln polymorphism and the Asp827Glu allele were more frequent in thyroid carcinoma patients than in healthy individuals, suggesting PTPRJ is a low-penetrance tumor susceptibility gene in human thyroid carcinogenesis (162). These mutations in the extracellular domain may affect the affinity for putative ligands, responding to stimuli differently and affecting downstream signaling pathways. It is also possible that these mutations may effect dimerization of DEP-1 in cis and alter signaling in this way as well. Functional assays with different allelic forms of PTPRJ will be required to further test the role of these polymorphisms.

DEP-1 in hematopoietic cells

An area of study that will not be covered in any detail in this thesis, but needs to be mentioned, is the role of DEP-1 in hematopoietic cells. DEP-1, or CD148, is widely expressed on the membranes of human hematopoietic cells and its expression is quite variable between cell lineages (165-167). Flow cytometric studies have demonstrated especially strong expression of CD148 on granulocytes, monocytes/macrophage, and mature

thymocytes (165, 166). Weaker expression was found on peripheral blood lymphocytes, T cells, B cells, platelets, natural killer cells, and dendritic cells (165, 167), and poor expression was on transformed lymphoid T- and B- cell lines (165). Upregulation of CD148 occurred on T cells following activation (165, 166, 168, 169). Experiments in T cells have demonstrated a role for CD148 in the negative regulation of TCR signaling. Increased expression of CD148 reduced the activation of the TCR-induced transcription factor NFAT (nuclear factor of activated T cells) (169-171). In addition, CD148 can also act as a negative regulator by causing specific dephosphorylation of LAT (linker for activation of T-cell) and phospholipase Cγ1 (170). Unlike T cells, CD148 expression levels did not change substantially following stimulation and activation of B cells (167), suggesting that CD148 is not regulating activity of B cells in a similar manner as T cells.

Cross linking of CD148 can induce increased [Ca^{2+}] and tyrosine phosphorylation of several proteins in human T cells including phospholipase Cγ1 (165). The increased phosphotyrosine of intracellular proteins suggests that CD148 might regulate a protein tyrosine kinase whose activity is dependent on its phosphorylation status. The inhibitory tyrosine residuce of src has been shown to be a substrate of DEP-1 in malignant thyroid cells (172) and in B cells (173) and therefore could also be a substrate in T cells and other leukoctyes. The data above demonstrate that CD148 is involved in signal transduction in T cells and B cells, however, the significance of its action in immune response is still being evaluated. It is proposed that CD148 expression could be a marker for immune activation but further studies will need to be conducted to fully understand its role in hematopoietic cells.

Tight Junctions

Protein components of Tight Junctions

Tight junctions are the most apical of the junctions formed by epithelia and provide a regulated barrier to paracellular transport of ions, solutes, water as well as cells. They function as a fence, preventing the mixing of membrane proteins between the apical and basolateral membranes. Tight junctions consist of 4 transmembrane proteins and several cytoplasmic proteins which anchor the transmembrane proteins to the actin cytoskeleton (Fig 1.5). Selected protein-protein interactions are depicted in Table 4. In the transmembrane group there are the junctional adhesion molecules (JAMs), occludin, tricellulin, and claudins (174-176). My work has focused on occludin, therefore I will not go into great detail about the other transmembrane proteins but will instead briefly introduce them. Several review articles cover them in more depth (177-181). The JAMs have 3 family members, A, B, and C. They localize to TJ and engage in both homophilic and heterophilic adhesions, however do not induce the formation of TJ strands in cells lacking TJ (182). Tricellulin is very similar in structure to occludin, but is only located at tricellular junctions and strengthens the barrier function of epithelial cell sheets (176). The claudin family consists of 24 members with each exhibiting distinct expression patterns in both tissue- and cell- specific manners (reviews see (175, 183, 184). Also similar in structure to occludin but not in sequence (185), claudins are capable of forming TJ strands when expressed in L cells, fibroblasts normally lacking TJs (186). Claudins are believed to be the main TJ component driving the overall structure of TJ and the variety in strength, size, and ion specificity of tight junctional barriers in different

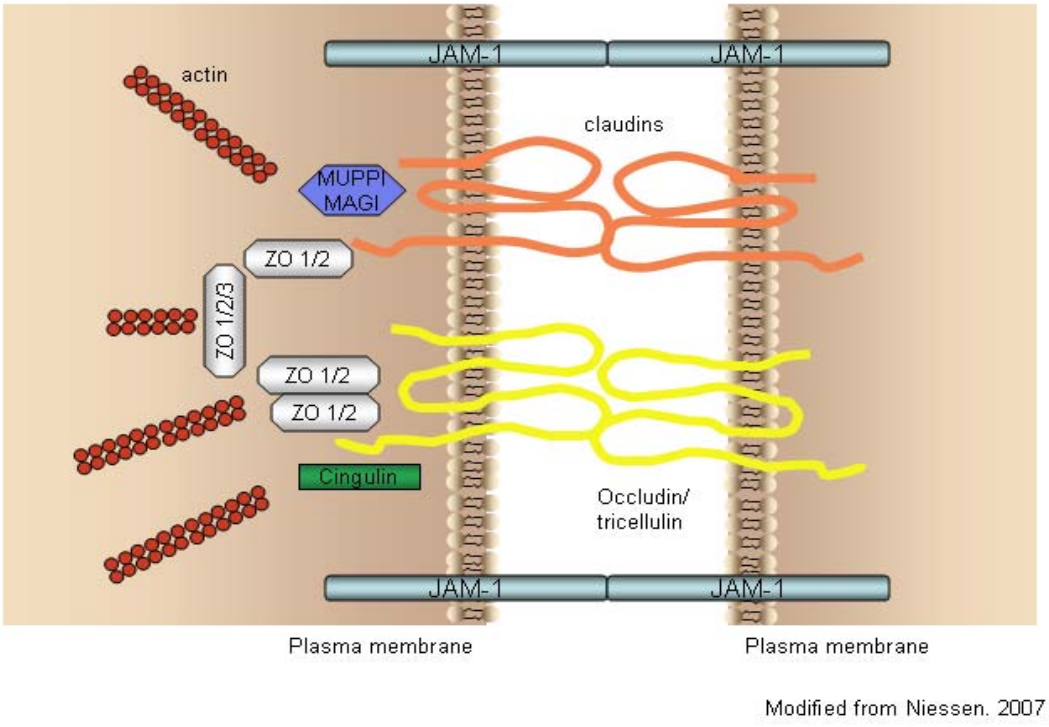


Figure 1.5. Schematic representation of the basic components of tight junctions. Claudins and occludin/tricellulin span the membrane four times and have N- and C-terminal cytoplasmic domains. Tricellulin is only localized at tricellular junctions. Junctional adhesion molecules (JAMs) are IgG-like domain containing proteins which can form homophilic or heterophilic interactions at junctions. ZO-1 or ZO-2 is important for clustering of claudins and occludin, resulting in the formation of tight junctional strands. The role of the other scaffolding proteins (ZO-3/MAGI/MUP1) is less clear. The ZOs and cingulin can provide a direct link to the actin cytoskeleton. Modified from (178).

Table 4. Tight Junction proteins interact with several other TJ proteins

TJ PROTEINS	OTHER TJ BINDING PARTNERS
Occludin	ZO-1,-2,-3, cingulin
Claudins	ZO-1, -2, -3, MUPP1
ZO-1	Occludin, claudins, ZO-2, ZO-3, cingulin, Ga12, F-actin
ZO-2	Claudins, ZO-1, cingulin, F-actin
ZO-3	Claudins, ZO-1, F-actin

Modified from Matter et al. Curr Opin Cell Biol (192)

epithelia and endothelia is largely due to the type of claudin(s) found at specific tight junctions (175, 187).

Occludin spans the membrane four times creating two extracellular loops with both an N- and C-terminal cytoplasmic tail and was the first transmembrane component of the TJ to be identified (188). Early studies of both full length and mutant occludin in epithelial cells (MDCK) or Xenopus initially suggested a role for occludin in the barrier and fence function of TJs (189, 190). Truncation and overexpression studies have shown that the N-terminal domains (intracellular N-terminal tail as well as 2 extracellular loops) are sufficient for localization of occludin to the the TJ when endogenous occludin is already present (189-191), and for the maintenance of barrier properties of epithelia (193). The extracellular domains interact in a homophilic manner and form adhesive contacts with proteins on adjacent cells (186). Transfection of occludin into fibroblasts lacking TJs was able to induce Ca2+-independent adhesions. Addition of synthetic peptides or antibodies corresponding to the extracellular domains of occludin disrupted adhesions, resulting in loss of occludin at the membrane and perturbing the barrier function of epithelial monolayers (194, 195). Therefore, interaction of the extracellular domains of occludin may be a method by which occludin can form an intercellular seal.

There is also data supporting the importance of the C-terminal tail in mediating occludin localization at TJs. The C-terminal tail can be divided into two distinct subdomains. The subdomain comprising the 150 amino acids proximal to the membrane is not highly charged, and is less conserved across species compared to other domains of occludin (196). In addition, it is not known to interact with other proteins. However, the domain encompassing the last 150 amino acids (ZO-binding domain) is highly charged, relatively

conserved across species, and binds directly to F-actin, cingulin, ZO-1, ZO-2 and ZO-3 (197-202). Loss of this ZO-binding domain prevents occludin localization to TJs in a bovine kidney cell line (198) suggesting its importance in targeting to TJs. A connexin-occludin chimera containing the ZO-binding domain and not the membrane proximal domain of occludin was able to localize at TJs when ZO-1 is present in cells (203). Combined these studies suggest the importance of this domain and for the role of cytoplasmic protein binding (ZO family in particular) in localizing occludin at TJs. Although there is evidence to suggest that occludin is important for TJ barrier and fence function, occludin knockout mice still had epithelia with TJ strands and intact barrier function (204). The mice do have mild phenotypes including growth retardation, male sterility and gastritis suggesting some level of barrier impairment (204). Perhaps occludin is more of a regulatory player in the TJ and not essential to its formation. The exact physiological function of occludin remains unclear.

The transmembrane proteins of the TJ do not directly interact with one another; therefore, there are a number of cytoplasmic proteins that link the transmembrane proteins to each other and to the actin cytoskeleton. These include the ZO family of proteins, cingulin and MUPP1/MAGI proteins (Fig. 1.5). I will not go into great detail on cingulin and MUPP1/MAGI, however it is noteworthy that they are localized at the TJ cytoplasmic plaque and are scaffolding proteins, linking one or more transmembrane proteins with the actin cytoskeleton (174). There are 3 ZO proteins, ZO-1, ZO-2, and ZO-3. They are members of the MAGUK family (membrane-associated guanylate kinase-like homologs). This family is characterized by N-terminal PDZ domains, a SH3, a GUK domain, an actin binding region, and a C-terminal proline rich region (205). ZO-1 interacts with JAMs, forms homo- or heterodimers with ZO-2 and ZO-3 (201), and also directly interacts with claudins via binding

of the PDZ domains to the C-terminal tail (200). ZO-1 localization to TJ requires the actin-binding domain (206) as well as the SH3-U5-GUK-U6 region (207). Point mutants in the SH3 domain of ZO-1 have showed that the SH3 domain is important in regulating TJ assembly of epithelial cells (208). ZO-1 and ZO-2 are also necessary for clustering claudins, TJ strand formation and barrier function (209).

Tyrosine phosphorylation of Tight Junctions

As mentioned previously in this chapter, increased tyrosine phosphorylation of AJ proteins decreases junctional integrity and decreases the barrier properties of epithelial and endothelial cells (69-71). Components of the TJ are also regulated by tyrosine phosphorylation, including claudins, afadin, occludin, and ZO-1 (210, 211, Lohmann, 2004 #560, 212,Van Itallie, 1995 #500). I will discuss phosphorylation with regards to occludin and ZO-1.

PTP inhibitor studies have demonstrated that phosphorylation of ZO-1 and occludin and reduced transepithelial electrical resistance (TER) of the monolayer were associated with reduced PTP activity (212, 213). Oxidative stress and HGF treatment have also been shown to compromise barrier integrity and increase phosphorylation of both ZO-1 and occludin, which decreases their association with each other as shown by co-immunprecpitation and GST pulldown studies (214-216). HGF treatment also prevents ZO-1 from targeting to the tight junction (215). In addition, c-Src was found to bind to occludin and phosphorylate it. In vitro binding assays showed that when GST-c-terminal occludin was phosphorylated by c-Src, significantly less ZO-1, ZO-2, and ZO-3 bound occludin compared to the non-phosphorylated c-terminal occludin(217). Strengthening of the junctions with cyclic-strain

produced opposite effects, with reduced permeability and occludin phosphorylation as well as increased occludin and ZO-1 localization to cell junctions (218). These studies suggest that increased tyrosine phosphorylation at TJs is associated with junctional disassembly and disruption of barrier function. They also support reports suggesting that tyrosine phosphorylated TJ proteins are often found in the soluble pool and non-phosphorylated forms are believed to be associated with the insoluble pool of proteins (219). There are a few conflicting reports dealing with phosphorylation of the TJ. Investigations have demonstrated that increases in phosphorylation can be associated with TJ assembly following ATP or Ca2+ repletion (219, 220). In the case of the calcium repletion study, removing the calcium decreased occludin tyrosine phosphorylation within 2 minutes and this correlated with a reduction in TER. Upon adding back calcium, tyrosine phosphorylation of occludin increased in parallel with an increase in TER, indicating occludin tyrosine phosphorylation is linked to TJ formation (220). These differences may have to do with different cell types used or the possibility that different stimuli may phosphorylate different residues.

*C*onclusions

PTPs play critical roles in cell-matrix adhesion dynamics as well as a central role regulating tyrosine phosphorylation in cell-cell adhesions. With respect to RPTPs, the interactions of their extracellular domains command considerable interest due to their similarity with cell adhesion molecules. Identifying their ligands is a high priority, as is determining whether these interactions regulate PTP activity. The large number of PTPs complexing with, and acting on, cadherins and their associated proteins is striking. Inhibiting PTP activity and elevating tyrosine phosphorylation at AJs and TJs promotes junctional

disassembly and affects TJ permeability. Together these observations demonstrate the importance of maintaining low tyrosine phosphorylation of junctional components in order to maintain normal epithelial and endothelial barrier functions. An exciting corollary of this is that agents that increase permeability or cells that need to cross these junctions, such as leukocytes or tumor cells, may modulate cell junctions by manipulating PTPs. Exploring this possibility is one of the challenges facing this field.

The effect of tyrosine phosphorylation on TJ proteins remains unclear. Perhaps phosphorylation can be involved with both assembly and disassembly, depending on different tyrosine residues in relevant proteins. This has been shown for focal adhesions, with phosphorylation of FAK leading to new binding sites for SH2 domain-containing proteins. When phosphorylated, Tyr 397 interacts with the SH2 domain of Src leading to increased phosphorylation and the disassembly of focal adhesions. The possibility also exists that tyrosine phosphorylation may affect junction permeability in a distinct way compared to its role in regulating assembly and disassembly of junctions. Unfortunately, not much is known about this. Mapping the phosphorylation sites and correlating changes in the phosphorylation states of the substrates with regards to assembly, disassembly, and altered permeability will eventually lead to a better understanding of what tyrosine phosphorylation is regulating, however this is beyond the scope of this thesis. The next several chapters detail the finding that tight junction proteins and focal adhesion proteins are substrates for the RPTP DEP-1. Characterizations of their interactions and consequences of their binding are discussed.

Chapter 2:

DEP-1 regulates phosphorylation of tight junction proteins and enhances barrier function of epithelial cells

Summary

Cell-cell adhesion is a dynamic process that can activate several signaling pathways. These signaling pathways can be regulated through reversible tyrosine phosphorylation events. The level of tyrosine phosphorylation of junctional proteins reflects the balance between protein tyrosine kinase and protein tyrosine phosphatase activity. The receptor tyrosine phosphatase DEP-1 (CD148/PTP-η) has been implicated in cell growth and differentiation as well as in regulating phosphorylation of junctional proteins. The effect of tyrosine phosphorylation on the integrity of cell-cell junctions is still under investigation. In this study, we used a catalytically dead substrate-trapping mutant of DEP-1 to identify potential substrates at cell-cell junctions. We have shown that in epithelial cells the trapping mutant of DEP-1 interacts with the tight junction proteins occludin and ZO-1 in a tyrosine phosphorylation-dependent manner. In contrast, PTP-PEST, Shp2, and PTPμ did not interact with these proteins, suggesting that the interaction of DEP-1 with occludin and ZO-1 is specific. In addition, occludin and ZO-1 were dephosphorylated by DEP-1 but not these other phosphatases *in vitro*. Over-expression of DEP-1 increased transepithelial electrical resistance in confluent epithelial monolayers and also reduced paracellular flux of FITC-dextran following a

calcium switch. These data suggest that DEP-1 can modify the phosphorylation state of tight junction proteins and play a role in regulating permeability and junction formation.

Introduction

Tight junctions are the most apical of junctions formed by epithelia and provide a regulated barrier to paracellular transport of ions, solutes, macromolecules, and even other cells. In addition, tight junctions act as a "fence" within the plane of the membrane, dividing the apical and basolateral domains of polarized epithelial cells. These junctions play an important role in the regulation of multiple cellular processes including cell differentiation, proliferation and polarity (see reviews (192, 221)). Functional tight junctions are characterized by the presence of membrane spanning proteins (claudins, occludin, and JAMs,) as well as cytoplasmic proteins (AF-6 and ZO-1,2,3). Occludin spans the membrane four times and was the first transmembrane component of the tight junction to be identified (188). It has two extracellular regions, an intracellular loop, as well as both an N and C-terminal cytoplasmic tail (188). The C-terminal tail of occludin binds directly to the ZO family of proteins, which link the protein complex to the actin cytoskeleton (197-199, 201, 203). The long C-terminal domain is rich in serine, threonine, and tyrosine residues, which can be phosphorylated by various kinases (174). Tyrosine phosphorylation of occludin is associated with a decrease in transepithelial electrical resistance (TER) (212, 222) and loss of protein localization at the tight junction (223). Increases in tyrosine phosphorylation of occludin and ZO-1 result in the dissociation of the occludin-ZO-1 complex and reduces these proteins localization at the tight junction (214, 217). These data suggest that the

phosphorylation state of tight junction proteins can affect the integrity of the tight junction complex and therefore the integrity of the tight junction itself.

Similarly, the other major junction of epithelia, the adherens junction (AJ), is regulated by tyrosine phosphorylation. Increased tyrosine phosphorylation of the AJ decreases the stability of the cadherin-catenin complex, disrupting the association with the cytoskeleton and reducing junctional integrity (70, 73, 74). Maintenance of junctional integrity is regulated in part by reversible tyrosine phosphorylation that results from a competing balance of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) activity. Several PTPs have been localized to AJs and shown to bind components of the cadherin-catenin complex. The PTPs in AJs include receptor-PTPs (PTPμ, DEP-1, and VE-PTP), as well as cytosolic PTPs (PTP1B and Shp-2) (78-83). The high concentration of PTPs, both cytosolic and transmembrane, at cell-cell junctions indicates the importance of maintaining low levels of tyrosine phosphorylation at these sites, except when the junctions need to be remodeled or disassembled.

DEP-1 (density enhanced phosphatase-1) is a receptor PTP that was first cloned out of a human cDNA library and whose expression was elevated with increasing cell density (89). Also known as PTP-η, PTPRJ, and CD148, DEP-1 is comprised of an extracellular domain of eight fibronectin type III repeats, a transmembrane domain, and a single cytoplasmic catalytic domain. The protein is ubiquitously expressed (143), suggesting it's involvement in a large number of diverse signaling pathways. DEP-1 is involved in regulating the differentiation of epithelial cells (139, 141, 224, 225), as well as controlling cell growth and adhesion (139, 141). In addition, DEP-1 is able to attenuate the cellular response to growth factors through the preferential dephosphorylation of several growth

factor receptors, suggesting that DEP-1 can selectively dephosphorylate certain residues to more finely control signaling (92, 149, 152, 153, 226).

In addition to its role in proliferation and differentiation, DEP-1 can also localize to areas of cell-cell adhesion in endothelial and epithelial cells, overlapping with the AJ marker VE-cadherin in endothelia (90). Direct interaction with p120 catenin as well as other members of the catenin family also supports the idea that DEP-1 plays a role in regulating AJ protein phosphorylation (80, 149). In the current study, we investigated whether tight junction proteins are also substrates of DEP-1. We now demonstrate that the substrate-trapping mutant of DEP-1 interacts with the tight junction proteins occludin and ZO-1. The association of DEP-1 with occludin and ZO-1 is specific to DEP-1 and not other phosphatases tested. The binding to the trapping mutant combined with DEP-1's ability to dephosphorylate occludin and ZO-1 *in vitro* indicates that these tight junction proteins are direct substrates of DEP-1. Furthermore, increased expression of DEP-1 enhances barrier function as junctions reform following a calcium switch. These results imply a role for DEP-1 in regulating the phosphorylation state of tight junction proteins and in influencing junction permeability.

Results

Identification of DEP-1 substrates at the tight junction.

To explore the function of DEP-1 at cell junctions, we sought to identify potential substrates of DEP-1 at the tight junction. To accomplish this we used a substrate trapping mutant of DEP-1 in which the conserved aspartic acid in the WPD loop of the catalytic domain is mutated to an alanine (D1205A), which traps the substrate within the

phosphatase's catalytic domain (8). Cytoplasmic domains of DEP-1 wildtype (DEP-1 WT) and the substrate-trapping mutant (DEP-1 D/A) were expressed as GST fusion proteins and used in pulldown assays to identify potential physiological substrates of DEP-1. MCF10A cells were treated with pervanadate to generate a pool of tyrosine phosphorylated proteins. As seen in Fig. 2.1A, only the substrate trapping mutant (DEP-1 D/A) was able to bind to phosphorylated proteins and DEP-1 D/A only bound to a subset of the phosphorylated proteins found in the lysate. Interestingly, we noticed a band at approx 64-kDa that is able to bind DEP D/A without the addition of pervanadate. It is possible that this protein is the 64kDa serine/threonine kinase found constitutively associated with DEP-1 (227).

DEP-1 is known to localize and interact with proteins located at adherens junctions (80, 149). We wanted to examine whether DEP-1 can also bind to and dephosphorylate neighboring tight junction proteins. To identify potential substrates, we again performed the GST-DEP-1 pulldown assay and probed with antibodies to known junctional proteins. The tight junction proteins occludin and ZO-1 were able to bind to DEP-1 D/A in a phosphorylation-dependent manner (Fig. 2.1B). The PTK src and p120 catenin, substrates of DEP-1 (172,Holsinger, 2002 #175), were also able to bind to DEP-1 D/A (Fig 2.1B). However, no interaction was detected between DEP-1 and junctional proteins E-cadherin and AF-6, or the cytoskeletal protein moesin (Fig. 2.1B). We can detect an interaction between occludin and DEP-1 D/A with as little as 5 μg of the fusion protein and it increases in a dose-dependent manner (Fig. 2.1C). However, it requires 20 μg of DEP-1 D/A to detect ZO-1 interaction (Fig. 2.1C).

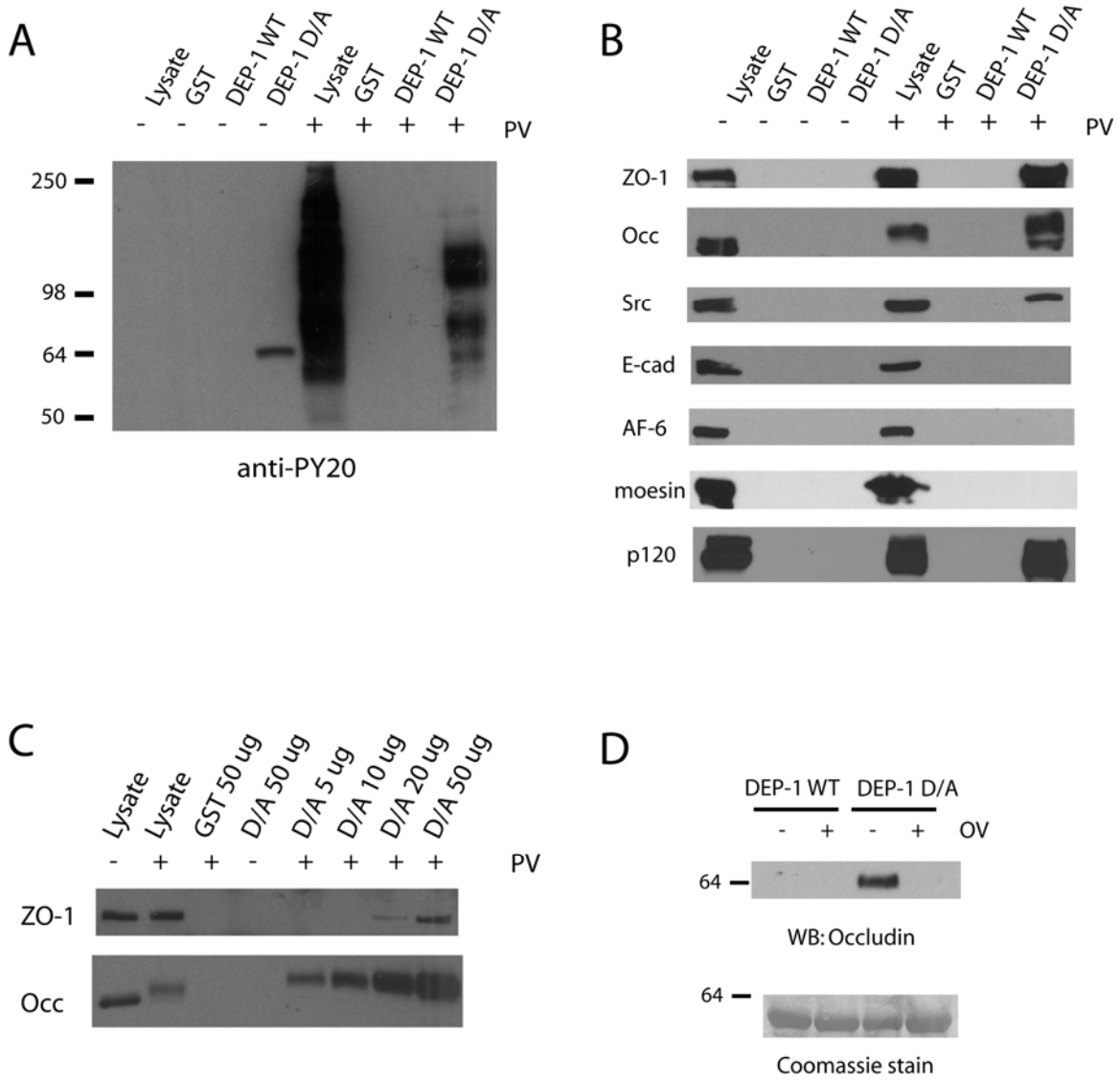


Figure 2.1. The substrate-trapping mutant of DEP-1 binds tight junction proteins in a tyrosine phosphorylation-dependent manner. MCF10A cells were either left untreated (-) or were treated (+) with 100 μM pervanadate for 10 mins prior to lysis. GST alone, GST-DEP-1 WT or GST-DEP-1 D/A fusion proteins were incubated with cell lysates and protein complexes were analyzed by SDS-PAGE and immunoblotting with ***A***. phosphotyrosine antibody (PY-20) or ***B***. antibodies to ZO-1, occludin, src, E-cadherin, AF-6, moesin and p120 ctn. ***C***. MCF10A cells were treated as described above. Lysates were incubated with the indicated amounts of GST proteins and protein complexes were analyzed by SDS-PAGE and immunoblotting with antibodies to ZO-1 and occludin. ***D.*** Effects of orthovanadate on the interaction between occludin and DEP-1 D/A. MCF10A cells were treated with pervanadate as described above. Cells were lysed with (+) or without (-) 2 mM sodium orthovanadate. GST-DEP-1 fusion proteins were preincubated with (+) or without (-) 2 mM sodium orthovanadate and added to the lysates. Protein complexes were analyzed by SDS-PAGE and immunoblotting with an occludin antibody. The membrane was stained with Coomassie blue to confirm that equal amounts of GST-DEP-1 proteins were used.

To determine if occludin directly binds to the catalytic site of DEP-1, the GST-DEP-1 proteins were pre-incubated with orthovanadate and tested for their ability to bind occludin. Orthovanadate is a competitive inhibitor that blocks the active site of DEP-1 and prevents substrate binding as well as enzymatic activity (228). In the presence of orthovanadate, the interaction between occludin and DEP-1 D/A was inhibited (Fig. 2.1D), indicating that occludin binds the active site of DEP-1's catalytic domain. These results suggest that DEP-1 specifically interacts with a small subset of junctional proteins that includes ZO-1 and occludin.

PDZ binding tail not responsible for DEP-1 interaction with ZO-1

The C-terminus of DEP-1 contains a type II PDZ binding tail with the last four amino acids being GYIA. Previously it has been shown that the four amino acid tail can bind to the PDZ domains of PDZ domain-containing proteins such as syntenin (229). ZO-1 contains 3 PDZ domains and we wanted to test whether the interaction between DEP-1 and ZO-1 or even DEP-1 and occludin were due to interactions with the PDZ binding tail. A truncation mutant of GST-DEP-1 D/A lacking the last four amino acids of DEP-1 (D/A Δ4) was used in a pulldown assay along with GST-DEP-1 D/A. The interactions between DEP-1 and ZO-1 or occludin were not affected by the loss of the last 4 amino acids of DEP-1 (Fig 2.2A and 2.2B). In addition, the binding of another known substrate, Src, was also not affected by loss of the last four amino acids (Fig 2.2B). This suggests that occludin and most importantly ZO-1 are not binding via interactions with the C-terminal tail of DEP-1 and are likely binding to the catalytic domain of the PTP.

DEP-1 dephosphorylates occludin and ZO-1.

Having shown that occludin and ZO-1 can bind to DEP-1, we wanted to further test whether they are in fact direct substrates of the phosphatase. First, MCF10A cells were treated with pervanadate to induce phosphorylation of occludin and ZO-1, and then lysates were incubated with GST, GST-DEP-1 WT, or GST-DEP-1 D/A. Occludin or ZO-1 was immunoprecipitated from the lysates and phosphotyrosine levels were revealed by blotting with an anti-phosphotyrosine antibody. As shown in Fig. 2.3A, wildtype DEP-1 dephosphorylated both occludin and ZO-1, whereas GST and DEP-1 D/A did not; these results confirm that occludin and ZO-1 are direct substrates of DEP-1 *in vitro*. Note the extensive band shift of immunoprecipitated occludin incubated with DEP-1 WT is consistent with dephosphorylation of occludin by the PTP. Next, we asked whether DEP-1 can dephosphorylate occludin when both are expressed together in cells. Full length DEP-1 WT or DEP-1 D/A were co-expressed with occludin in HEK 293ft cells, occludin was immunoprecipitated and phosphotyrosine levels were analyzed. In cells over expressing DEP-1 WT, phosphorylation of occludin was dramatically reduced compared to cells expressing empty vector or DEP-1 D/A (Fig. 2.3B). These combined experiments suggest that both ZO-1 and occludin are substrates of DEP-1 and that DEP-1 catalytic activity is required for the dephosphorylation of occludin in vivo.

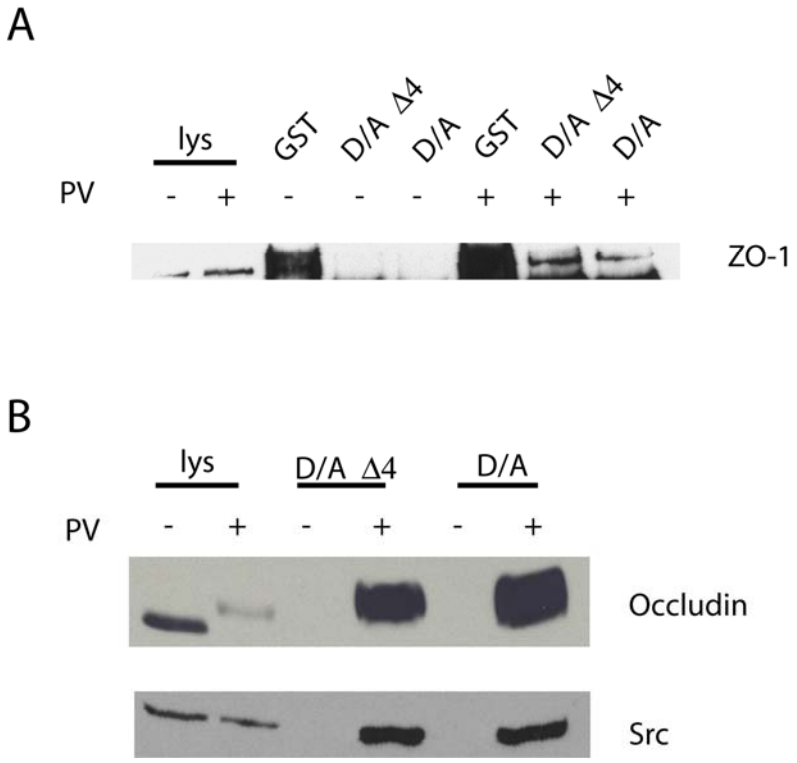


Figure 2.2. The PDZ tail of DEP-1 is not responsible for the interactions with substrates. A truncation mutant of DEP-1 missing the last four amino acids of the C-terminal tail was expressed as a GST fusion protein (GST-DEP-1 D/A Δ4) and tested for interaction with DEP-1 substrates. MCF10A cells were either left untreated (-) or were treated (+) with 100 μM pervanadate for 10 mins prior to lysis. Lysates were incubated with GST, GST-DEP-1 D/A (D/A), or GST-DEP-1 D/A Δ4 (D/A Δ4) and protein complexes were resolved with SDS-PAGE and immunoblotting with ***A***. ZO-1 antibody or ***B***. Occludin and Src antibodies. The smear in the GST lanes of (***A***) appear to be the non-specific binding of proteins and was found in the entire length of the lane. The GST control was not shown in (***B***) but previous experiments have never shown occludin binding to GST

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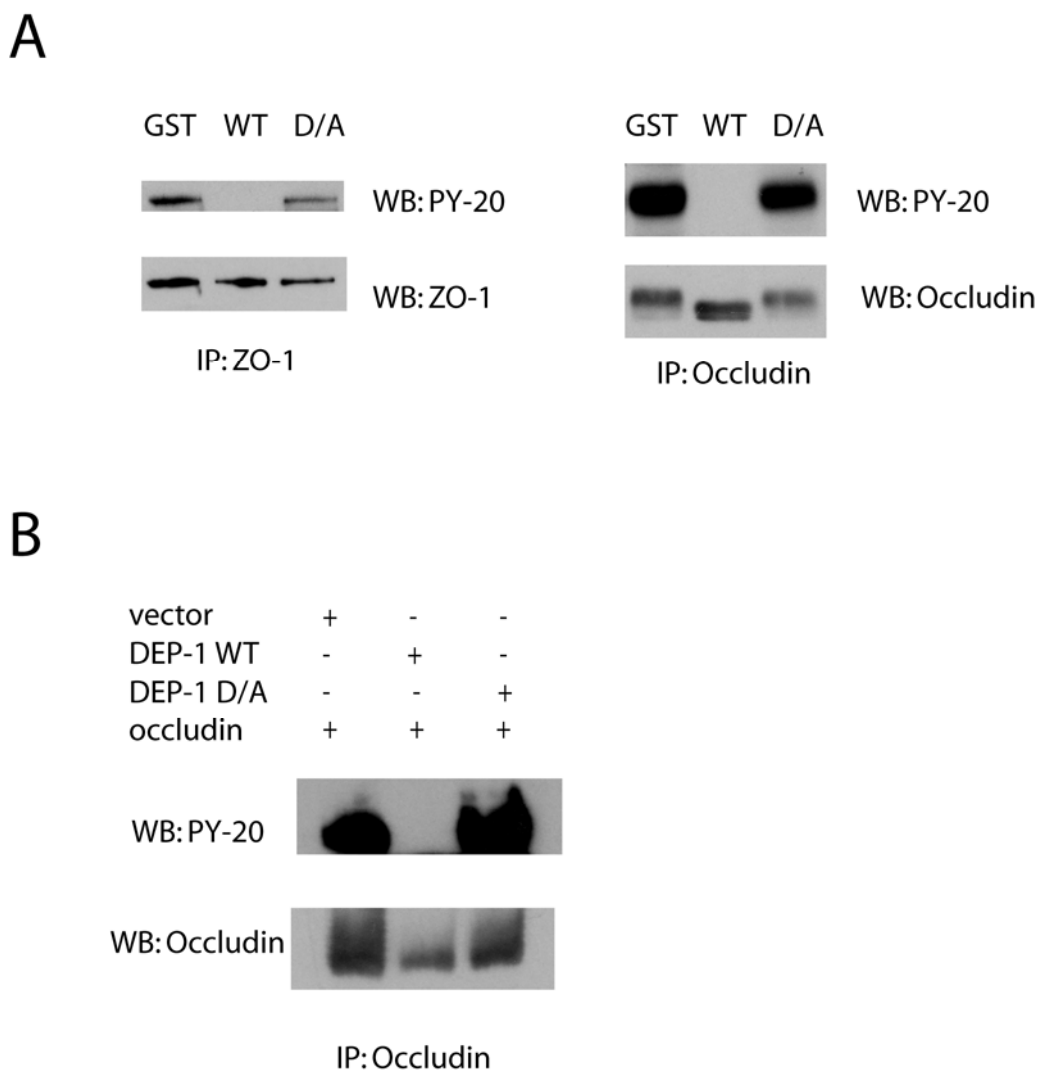

Figure 2.3. DEP-1 dephosphorylates occludin and ZO-1. ***A.*** In vitro dephosphorylation assay. MCF10A cells were treated with 100 μM pervanadate for 10 mins prior to lysis. Lysates were incubated with glutathione sepharose bound GST or GST-DEP-1 fusion proteins and then the fusion proteins were removed by centrifugation. Occludin and ZO-1 were immunoprecipitated from the remaining lysates. Immunocomplexes were analyzed by SDS-PAGE and immunblotted with PY-20 antibody or occludin/ZO-1 antibodies. ***B***. In vivo dephosphorylation assay. HEK 293ft cells were transfected with occludin and empty pMT2 vector or pMT2.DEP-1 WT or pMT2.DEP-1 D/A. Cells were treated with pervanadate (as in ***A***), lysed and immunoprecipitated with an anti-occludin antibody. Samples were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody or anti-occludin antibody.

Occludin and ZO-1 interaction is specific to DEP-1.

We wanted to determine if the interaction of DEP-1 with occludin and ZO-1 was specific or if these proteins were substrates of additional PTPs. Lysates of untreated or pervanadate treated MCF10A cells were incubated with GST fusion proteins of the substrate trapping mutants of DEP-1 (GST-DEP-1 D/A), PTP-PEST (GST-PTP-PEST D/A), and other junctional PTPs including Shp2 (GST-Shp2 C/S), and PTPμ (GST-PTPμ D/A). DEP-1 D/A was the only PTP of those tested that was able to interact with ZO-1 and occludin, and it did so in a tyrosine phosphorylation-dependent manner (Fig. 2.4A, 2.4B). To confirm that the substrate-trapping mutants were functional, pulldowns were blotted with an anti-phosphotyrosine antibody, and all were found to bind tyrosine-phosphorylated proteins (Fig. 2.5). In addition, membranes were stained with coomassie blue to confirm the use of equal amounts of PTP fusion proteins (Fig 2.4B). We also confirmed that other PTP's were comparatively inefficient at dephosphorylating occludin and ZO-1. MCF10A cells were treated with pervanadate to induce protein phosphorylation and incubated with wildtype GST fusion proteins of DEP-1, PTP-PEST, PTPα, and PTPμ. Following incubation, fusion proteins were removed by centrifugation, and then occludin and ZO-1 were immunoprecipitated and phosphotyrosine levels analyzed. As shown in Fig. 2.4C and 2.4D, GST-WT DEP-1 was able to completely dephosphorylate occludin and ZO-1 whereas the other PTPs had little effect. Again, notice the decrease in molecular weight of the occludin that accompanied dephosphorylation by DEP-1 WT (Fig. 2.4C). These results suggest that occludin and ZO-1 are specific substrates of DEP-1 and not other PTPs tested.

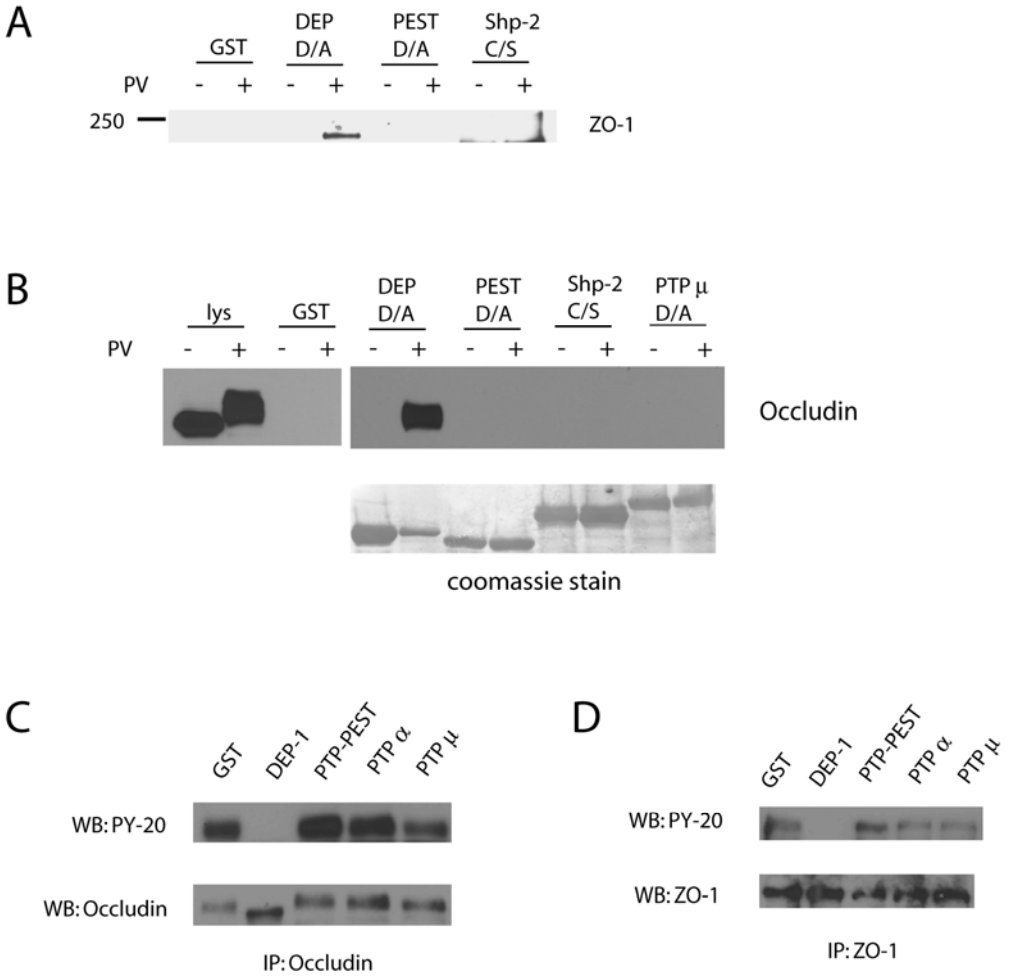

Figure 2.4. Occludin and ZO-1 are specific substrates of DEP-1. ***A & B***. Substrate-trapping mutants of different PTPs. MCF10A cells were either left untreated (-) or were treated (+) with 100 μM pervanadate for 10 mins prior to lysis. Lysates were were incubated with the GST-fusion proteins of the substrate trapping mutants of the indicated cytoplasmic and receptor PTPs. Protein complexes were analyzed by SDS-PAGE and immunoblotted for ***A***, ZO-1 and *B*, occludin. Membranes were stained with coomassie blue to confirm equal amount of PTP fusion proteins were used. ***C & D***. In vitro dephosphorylation assay. MCF10A cells were treated with 100 μM pervanadate for 10 mins prior to lysis. Lysates were incubated with GST or wildtype GST-PTP fusion proteins bound to glutathione sepharose, the fusion proteins were removed by centrifugation and ***C***, occludin or ***D***, ZO-1 were immunoprecipitated from the lysates. Immunocomplexes were analyzed by SDS-PAGE and immunblotted with a PY-20, occludin, or ZO-1 antibody.

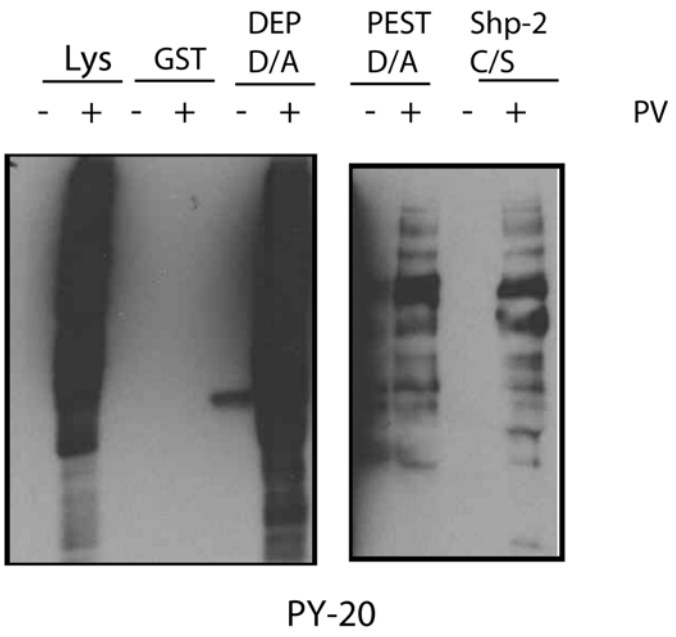

Figure 2.5. Substrate trapping mutants of PTPs are able to bind tyrosine phosphorylated proteins. MCF10A cells were either left untreated (-) or were treated (+) with 100 μM pervanadate for 10 mins prior to lysis. Lysates were incubated with GST, GST-DEP-1 D/A, GST-PTP-PEST D/A, and GST-Shp2 C/S for 1 hour and protein complexes were resolved with SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody (PY20). The two panels represent two exposure lengths. The PTP-PEST (-) PV lane has contaminating signal from the DEP-1 D/A (+) PV lane adjacent to it.

SAP-1 can dephosphorylate occludin

SAP-1 (stomach cancer-associated protein tyrosine phosphatase-1) is a receptor PTP which is structurally similar to DEP-1 with an extracellular domain of 8 fibronectin type III repeats, a transmembrane domain and a single intracellular catalytic domain (230). With its known structural similarity to DEP-1, we were interested in determining whether the tight junction proteins occludin and ZO-1 are substrates of SAP-1. We tested the catalytically inactive mutant of SAP-1 C/S in GST fusion pulldowns and found that occludin did not bind to this phosphatase compared to DEP-1 D/A (Fig. 2.6A, 2.6B). In addition, ZO-1 was not able to be pulled down with SAP-1 C/S (Fig. 2.6B).

We also examined the ability of SAP-1 to dephosphorylate occludin in an in vitro dephosphorylation assay. MCF10A cells were treated with pervanadate and the lysates were incubated with GST, GST DEP-1 WT or GST SAP-1 WT. Occludin was immunoprecipitated from the lysates and phosphotyrosine levels were determined by immunoblotting with a phosphotyrosine antibody. SAP-1 was able to visibly dephosphorylate occludin and appears to do so even better than DEP-1 (Fig 2.6C). Both PTPs have a dose dependent affect on phosphotyrosine levels. The preliminary results suggest that occludin may in fact be a substrate for SAP-1. This would not be all together surprising based on the similarities between the two PTPs.

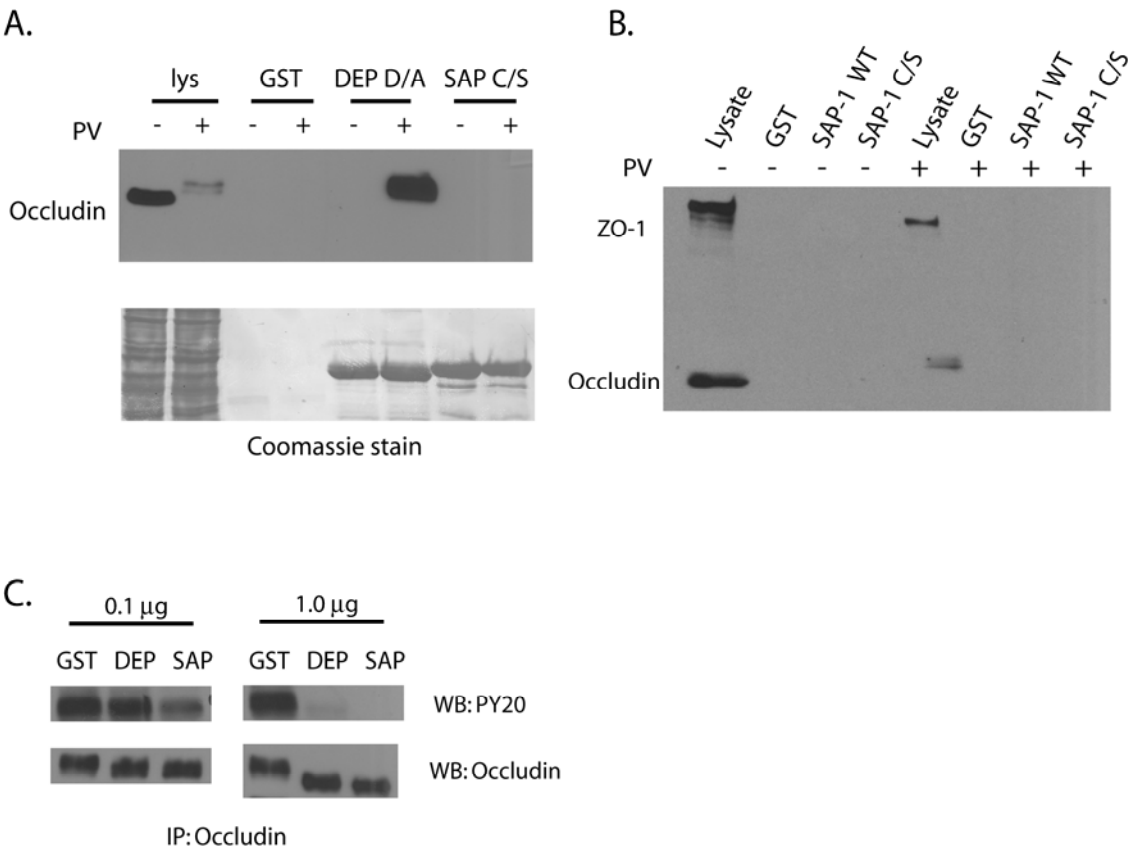

Figure 2.6. SAP-1 cannot bind occludin and ZO-1 but can dephosphorylate occludin. ***A***. MCF10A cells were either left untreated (-) or were treated (+) with 100 µM pervanadate for 10 mins prior to lysis. Lysates were incubated with GST, GST-DEP-1 D/A or GST-SAP-1 C/S. Protein complexes were resolved with SDS-PAGE and immunoblotting with an occludin antibody. The membrane was also stained with coomassie blue to ensure equal amounts of fusion proteins were used. ***B***. Lysates were prepared as in ***A***. and incubated with GST, GST-SAP-1 WT and GST-SAP-1 C/S. Membranes were probed with ZO-1 and occludin antibodies and neither were found to interact with the SAP-1 proteins. ***C***. In vitro dephosphorylation assay. MCF10A cells were treated with 100 µM pervanadate for 10 mins prior to lysis. Lysates were incubated with GST or wildtype GST-DEP-1 and GST-SAP-1 bound to glutathione sepharose. The fusion proteins were removed by centrifugation and occludin was immunoprecipitated from the lysates. Immunocomplexes were analyzed by SDS-PAGE and immunblotted with a PY-20 or occludin antibody.

DEP-1 binds and dephosphorylates Src-induced phospho-occludin

There are 43 tyrosines in occludin, 23 of which are located in either the intracellular N- or C-terminus. In the previous experiments we used pervanadate to induce tyrosine phosphorylation of junctional proteins. Pervanadate is an irreversible PTP-inhibitor that is 10^2-10^3 times more potent than vanadate alone (Kadota, Posner 1987; Fatutus and posner 1989). This allows us to increase pools of tyrosine phosphorylated proteins, however, it is difficult to determine which kinase is acting on our substrates or which residues are phosphorylated. The identification of kinase(s) acting on the tight junction proteins as well as the tyrosine residues that DEP-1 is binding would help to clarify how tyrosine phosphorylation is regulating tight junction function. One potential kinase is Src kinase. Src localizes to tight junctions (231), binds ZO-1 (232), and can phosphorylate occludin *in vitro* leading to disruption of TJs (217). There are several Src consensus sites in the N-and C-terminal tails of occludin and DEP-1 may be able to bind to one of these sites. First, we examined whether constitutively active Src (Y527F) was able to phosphorylate occludin in transfected cells. HEK 293ft cells were co-tranfected with full length occludin and either empty vector or pCMV-SrcY527F. Cells were lysed in hot SDS sample buffer, occludin was immunoprecipitated, and phosphotyrosine levels were analyzed. Expression of constitutively active Src was able to phosphorylate occludin (Fig. 2.7A), supporting data by Kale et al. (217). Next, we determined whether the Src induced phospho-occludin was able to bind to the substrate trapping mutant of DEP-1. Again, HEK 293ft cells were transfected with occludin and either empty vector or SrcY527F. GST-DEP-1 pulldown assays were performed with the lysates from the transfected cells and the membranes were probed for occludin. We found that Src-phosphorylated occludin interacted with the substrate trapping

mutant of DEP-1 (Fig. 2.7B). Knowing that Src can phosphorylate occludin and these phosphorylation sites interact with the substrate trapping mutant of DEP-1, we tested whether wildtype DEP-1 can dephosphorylate Src-induced phospho-occludin. We expressed wildtype or the catalytically inactive substrate trapping mutant of DEP-1 with occludin and SrcY527F in HEK 293 cells, immunoprecipitated occludin and analyzed phosphotyrosine levels. The addition of DEP-1 WT reduced the phosphotyrosine levels of occludin, suggesting that DEP-1 was able to dephosphoorylate Src phosphorylation sites (Fig. 2.7A). It is interesting to note that HEK 293ft cells have endogenous DEP-1 and when transfected with constitutively active Src, endogenous DEP-1 levels increase (Fig. 2.7A).

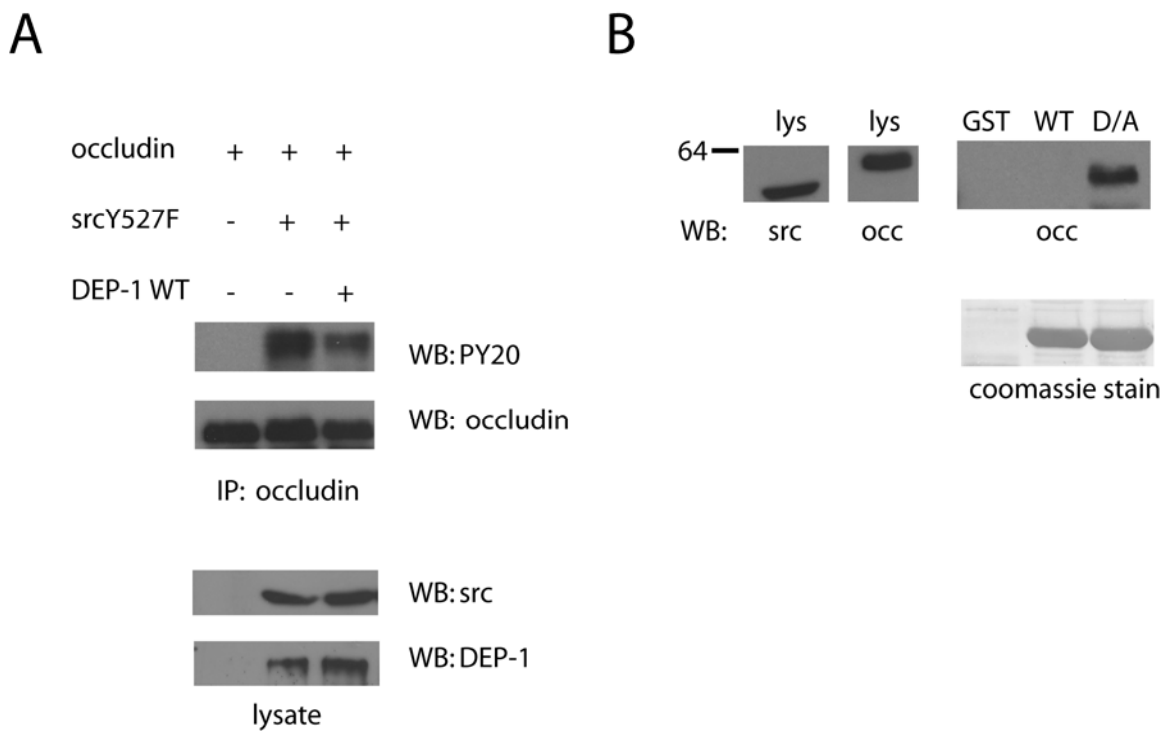

Figure 2.7. Src phosphorylation of occludin and induced interaction with DEP-1. ***A***. HEK 293ft cells were transfected with occludin, occludin and constitutively active Src (SrcY527F), or occludin, SrcY527F, and DEP-1 WT. Cells were lysed in hot 2X SDS gel sample buffer and samples were then diluted 20-fold with phospho-IP buffer to a final SDS concentration of 0.1%. Occludin was immunoprecipitated, IPs resolved by SDS-PAGE and membranes were probed with PY-20 or occludin. In addition, lysates were blotted for src and DEP-1 expression. ***B***. HEK 293ft cells were transfected with SrcY527F and occludin and lysates were used in a pulldown assay with GST, GST-DEP-1 WT and GST-DEP-1 D/A. Lysates and pulldowns were resolved by SDS-PAGE and immunoblotting for src and occludin expression as well as occludin binding to the fusion proteins. The membrane was subsequently stained with coomassie blue to ensure equal amounts of GST fusion proteins were used in each pulldown.

DEP-1 and occludin co-localize at areas of cell-cell contact.

Previous studies have shown that DEP-1 localizes at points of cell-cell contact as well as along the apical plasma membrane (80, 90). We wanted to examine to what extent DEP-1 and occludin co-localize along cell tight junctions. MDCK II cells were transfected with GFP or DEP-1 WT-GFP and were stained with anti-occludin antibodies to visualize endogenous occludin at tight junctions. As shown in Figure 2.8, there was significant colocalization of DEP-1 and occludin at sites of cell-cell contact.

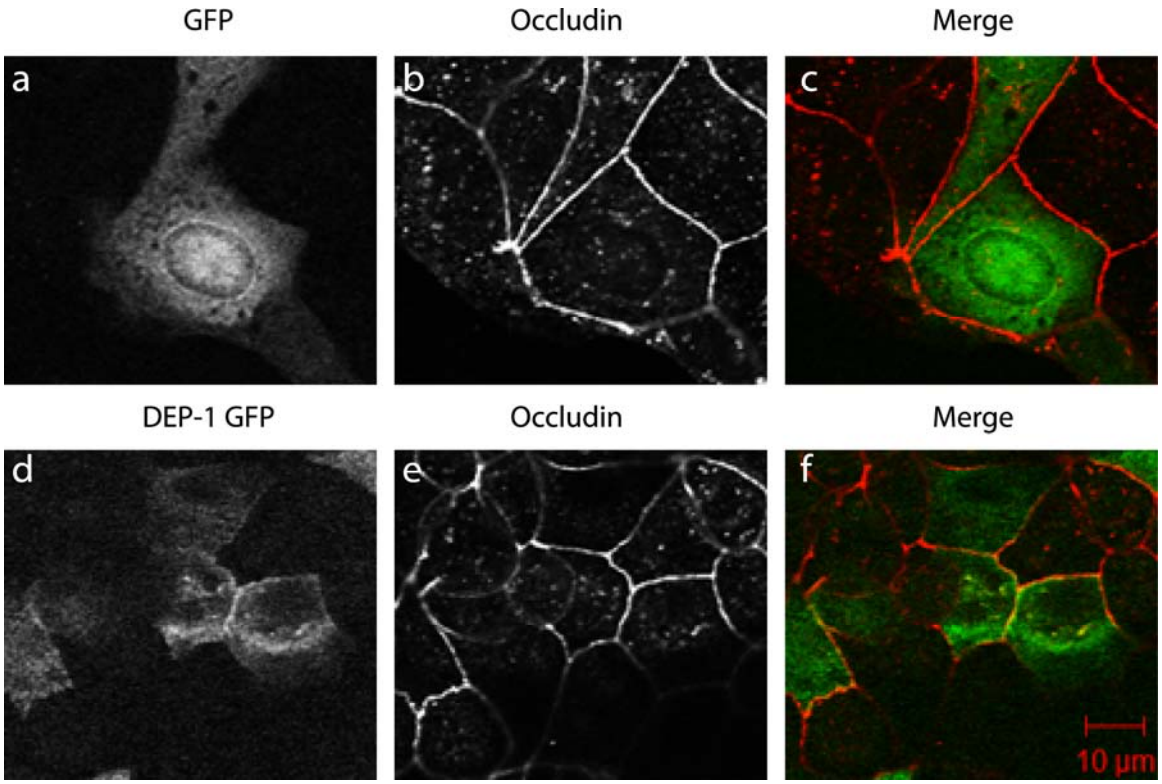

Figure 2.8. DEP-1 localizes to areas of cell-cell contact. MDCK II cells were transfected with pEGFP or pEGFP-DEP-1 (a,d) and stained for occludin (b,e). Merged images show colocalization in yellow (c,f). *Scale bar = 10 µm*.

DEP-1 expression enhances barrier function as junction reassemble.

We next wanted to determine whether our previously observed dephosphorylation of occludin and ZO-1 had functional consequences with respect to tight junction physiology. MDCK II cells were infected with GFP or DEP-1 WT adenovirus, plated onto transwell filters, and 4 days later the transepithelial electrical resistance (TER) of the stable junctions was measured. In epithelial cells overexpressing DEP-1 we saw a small but significant increase in TER compared to the GFP control ($p<0.005$) (Fig. 2.9), supporting the hypothesis that expression of DEP-1 can decrease the permeability of the tight junction.

To better address the ability of DEP-1 to regulate tight junction function, we looked at FITC-dextran flux across an epithelial monolayer during junctional reassembly following a calcium switch. MDCK II cells were infected with GFP or DEP-1 WT adenovirus and plated on transwell filters. After cells had adhered, growth media was replaced with low calcium (5 μM) media overnight. Normal calcium-containing media (1.8 mM) was then added to the transwells and the ability of FITC-dextran to pass across the monolayer was assessed at 0, 6, 12, 24 and 48 hours. The amount of FITC-dextran that crossed the monolayers was significantly reduced as junctions reassembled (Fig. 2.10A). At early stages of junction reassembly (6 hrs), the presence of additional DEP-1 WT decreased permeability indicated by the further decrease of FITC-dextran flux compared to GFP control cells (Fig. 2.10A, 2.10B.) After 12 and 24 hours in calcium-containing media, the permeability between GFP- and DEP-1 expressing cells was not significantly different (Fig. 2.10A, 2.10B). In identical experiments, TER was measured following the calcium switch. Monolayers over-expressing DEP-1 WT have higher TER than GFP controls at early time points of junction reassembly

(Fig. 2.10C). Together the TER and FITC-dextran data reveal that an increase in DEP-1 WT enhances the barrier function of

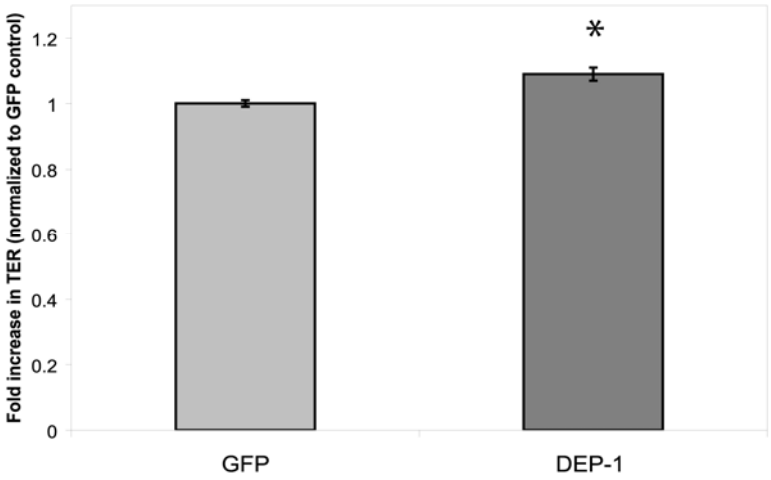

Figure 2.9. DEP-1 enhances junctional integrity in epithelial cells. MDCK II cells were infected with GFP alone or DEP-1 WT adenovirus, plated on filters (0.4μm pore) and allowed to grow into confluent monolayers. Transepithelial electrical resistance was taken at 4 days post plating. Values are expressed as the mean ± SEM of 4 experiments performed in triplicate. Baseline TER ranges from 45-50 ohms/cm$^{2.}$ *Asterisk* indicates the value is significantly ($p<0.005$) different from corresponding values for control group (GFP).

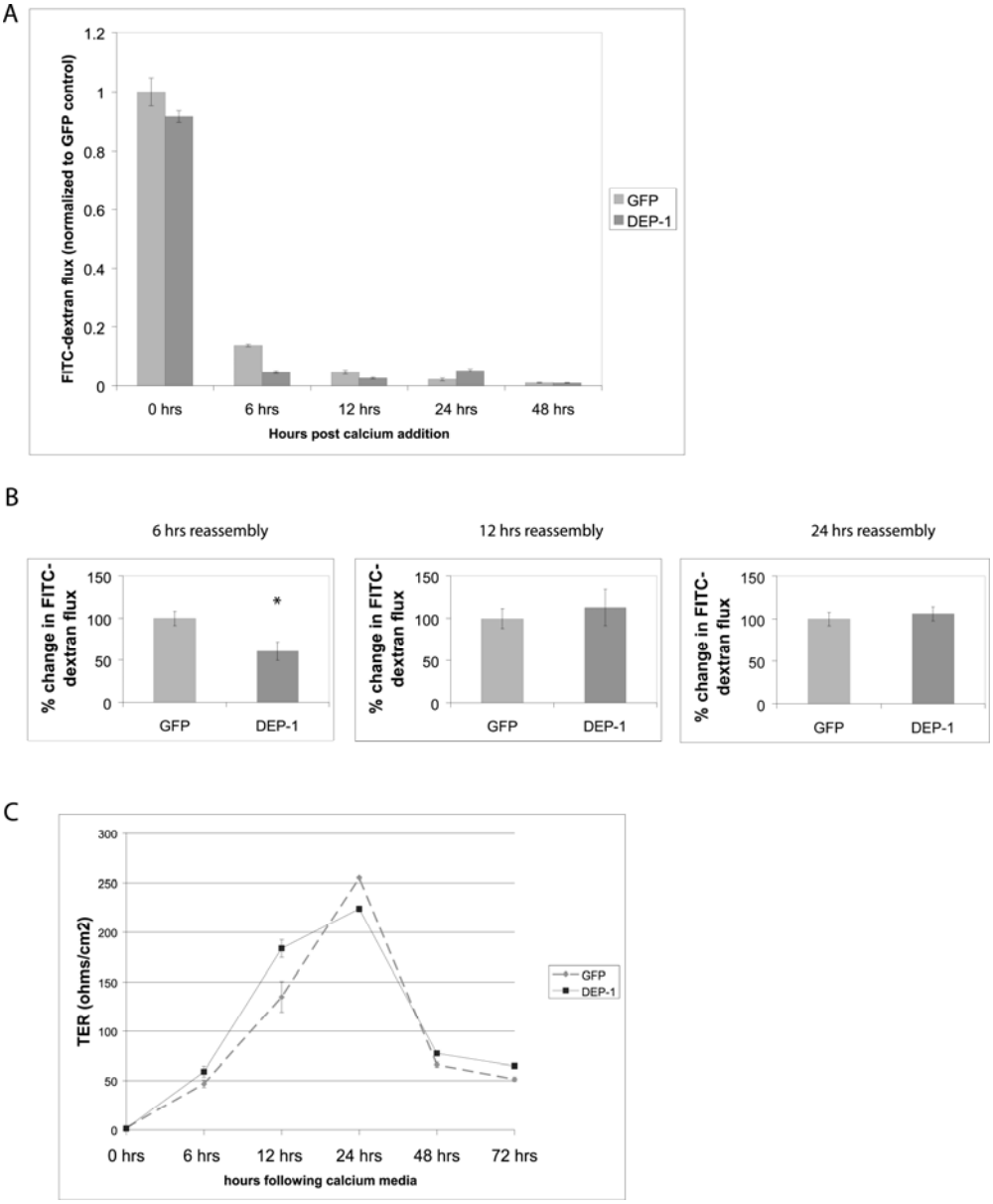

Figure 2.10. Expression of DEP-1 enhances barrier function as junctions reassemble. MDCK II cells were infected with GFP or DEP-1 WT adenovirus and grown on filters as confluent monolayers. Cells were incubated in low calcium media (5 μM) for ~16 hours to completely disrupt junctions (as measured by TER), and then transwell filters were switched to normal calcium media (1.8 mM). ***A***. At varying time points after the addition of calcium media, 10-kDa FITC-dextran was added to the top chamber of the transwell filter and allowed to cross the cell monolayer for one hour. Values across all times were normalized to the amount of FITC-dextran flux that crossed the GFP control cell monolayer at 0 hrs. The amount of FITC-dextran that crossed the monolayer was analyzed using a fluorometer (excited 485 nm, emission 520 nm). The graph shows a representative experiment. ***B***. To directly compare flux differences at specific time points, values were normalized to GFP controls at 6 hrs, 12 hrs and 24 hrs. Data are expressed as mean ± SEM of at least 3 experiments performed in at least duplicate. *Asterisk* indicates the value is significantly ($p<0.01$) different from corresponding values for control group (GFP). ***C***. TER was measured over 72 hours after the calcium switch. Data is expressed as mean ± SEM of filters in triplicate. This graph shows a representative experiment.

epithelial junctions during reassembly. Thus, the presence of DEP-1 is particularly important at the earliest, most dynamic stages of junctional assembly.

Discussion

Cell-cell junctions can be regulated through reversible tyrosine phosphorylation. Tyrosine phosphorylation of adherens junction proteins has been shown either to promote the dissociation of protein complexes at the junction (70, 73, 74, 77) or to promote the internalization and degradation of junctional proteins (76). Whereas most work in this area has focused on the tyrosine kinases that act on junctional proteins, it is equally important to identify the PTPs and their targets within cell-cell junctions. Here we show for the first time that the prominent tight junction proteins, occludin and ZO-1, interact with and are dephosphorylated by the PTP DEP-1. Experimentally increasing the levels of DEP-1 reduced the permeability of epithelial monolayers and this effect was enhanced during the early stages of junction assembly.

Similar to the adherens junctions, tyrosine phosphorylation has been correlated with the dissociation of tight junction components, promoting the detachment of ZO-1 from occludin (214, 215, 217, 233, 234). This increased tyrosine phosphorylation of tight junction components is paralleled by increased epithelial permeability. Because DEP-1 has previously been localized to regions of cell-cell contact (80, 90), we examined whether the phosphorylation of tight junction proteins was regulated by this PTP. We found that the catalytically dead trapping mutant of DEP-1 binds to phosphorylated forms of occludin and ZO-1 and that catalytically active DEP-1 dephosphorylates these proteins. Previous work has shown that DEP-1 interacts with and acts on the adherens junction proteins p120catenin

and β-catenin (80, 149), and we have confirmed these interactions (Fig. 2.1B and data not shown). However, we found that DEP-1 does not bind all tyrosine phosphorylated junctional proteins (Fig. 2.1B). It is striking that several PTPs are concentrated within epithelial junctions (235) and it is likely that there is some redundancy with respect to their targets. However, we were surprised to find that two junctional PTPs, PTPμ and SHP-2, did not significantly interact with or dephosphorylate occludin and ZO-1 (Fig. 2.4). This result suggests that the different PTPs within cell-cell junctions have specific targets and that they may regulate distinct signaling pathways. These pathways may either be initiated within junctions in response to cell-cell interactions or be triggered by a stimulus such as growth factors or oxidative stress to regulate junction stability, strength, and permeability. It is worthy of note that we discovered that the highly homologus PTP SAP-1 is able to dephosphorylate occludin although no interactions with the substrate trapping mutant (SAP-1 C/S) were found. The C/S mutation in PTP catalytic domains does not have as high an affinity for substrates since the mutation is of the catalytic cysteine. As a result, the C/S mutant is not as efficient at binding substrates as compared to the D/A mutant (8). Noguchi et al. (236) have specifically shown that SAP-1 C/S is less efficient at binding proteins from pervanadate treated cells than SAP-1 D/A. Therefore, the D/A mutant will be made in the GST-SAP-1 construct and this experiment will be repeated to clarify whether tight junction proteins may be substrates. This may provide evidence that although different types of PTPs may have different substrates, PTPs within the same family type may regulate similar signaling pathways.

Having found that DEP-1 dephosphorylates occludin and ZO-1, we wanted to explore DEP-1's effect on tight junction function in epithelial cells. Mature junctions are thought to

exist in a tyrosine dephosphorylated state, with increased tyrosine phosphorylation leading to increased junction disassembly, increase paracellular permeability and can induce reorganization of the junctional complex (212, 222). Thus, dephosphorylation of occludin as well as other junctional proteins, is believed to increase the barrier function of the junction. Our over-expression of DEP-1 in epithelial cells increased TER (Fig. 2.9), a measure of increased barrier properties of the junctions, consistent with phosphorylation regulating the permeability of tight junctions. Although the difference was subtle (5-10% increase), it was reproducible and statistically significant. We believe the small effect on TER is due to the fact that the cells had been confluent for several days. At this point, the junctions have reduced dynamics and the level of tyrosine phosphorylation is very low (essentially undetectable). Because tyrosine phosphorylation is elevated during disassembly/assembly of junctions, this led us to explore the effect DEP-1 had on junctional permeability as the junctions were assembling. Using the established calcium switch technique (237, 238) to modulate cell-cell adhesion, we measured the permeability of epithelial junctions with both TER and FITC-dextran flux over a time course of junction reassembly. As shown in Figure 2.10A, as junctions reassemble after calcium addition, the permeability decreases over time. Importantly, when comparing the permeability of GFP-expressing versus DEP-1-expressing cells at 6 hrs following calcium restoration, the presence of DEP-1 significantly reduces the permeability of FITC-dextran flux, suggesting that these junctions reassembled more rapidly (Fig. 2.10B). By 12 and 24 hours the difference in permeability between GFP- and DEP-1-expressing cells was not statistically significant (Fig. 2.10B). The TER data shows a slightly shifted trend, with the cells over-expressing DEP-1 still maintaining an increase in TER at 12 hrs following the initiation of reassembly. This difference may reflect the difference between

measuring the permeability of ions versus macromolecules in epithelial cells. Overall, our results suggest a model in which DEP-1 is a receptor PTP that localizes to cell-cell junctions, and is able to regulate phosphorylation levels of junctional proteins (Fig. 2.11). In this way, it aids in the reformation of the tight junction complex and enhances barrier function of epithelial junctions during Ca^{++}-induced reassembly.

DEP-1 was first identified as a PTP whose expression was enhanced with increasing cell density, suggesting its potential role in contact inhibition of growth (89). In recent years much work has been done to characterize the role of DEP-1 in cell proliferation and differentiation. DEP-1 can inhibit cell proliferation in a number of different cell types (140, 150, 164) and attenuates signaling downstream of growth factors by targeting the growth factor receptors themselves (92, 149, 152, 153). Studies have also found DEP-1 involved in cell differentiation (139, 162). Transformation of several cell types inhibited DEP-1 expression and reintroduction of the protein reduced growth and partially reverted the neoplastic phenotype (141, 142, 162). Frequent deletions, missense mutations, and loss of heterozygosity of DEP-1 have been found in a number of human cancers, including colon, lung and breast (163). These studies implicate the loss of DEP-1 as a factor in the disregulation of growth and differentiation in numerous tyrpes of cancers, and led to the proposal that it is a tumor suppressor. The action of DEP-1 on both adherens and tight junctions suggests another means by which DEP-1 may protect cells from oncogenic transformation and metastasis; controlling the strength and stability of cell-cell interactions. Diminished interaction of a cell with its neighbors is a hallmark of cancer and particularly associated with tumor invasion and metastasis. In future work, we hope to examine whether the relationship of DEP-1 with occludin and ZO-1 is changed in specific epithelial tumors.

The continued identification of junctional substrates of DEP-1 will aid in furthering our understanding of the role of this PTP in diseases such as cancer.

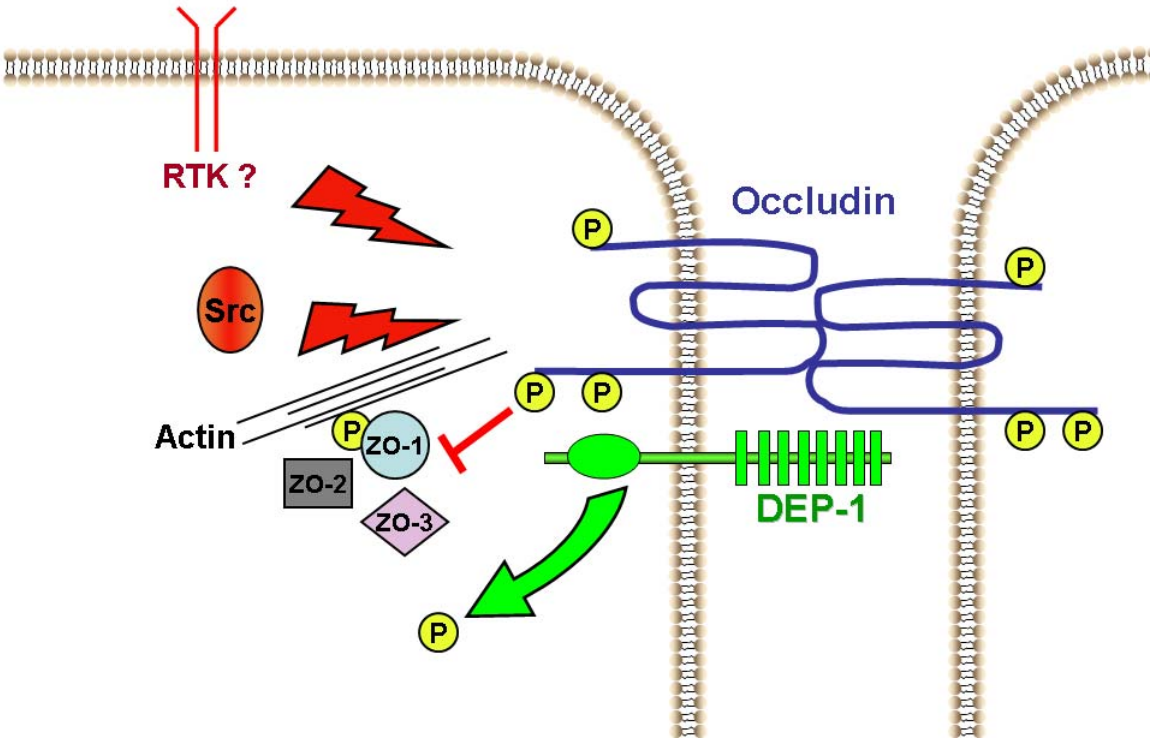

Figure 2.11. Model of how DEP-1 may regulate tight junction integrity. The transmembrane protein occludin binds via its C-terminal tail to the cytoplasmic ZO family of proteins. These ZO proteins link the complex to the actin cytoskeleton creating a stable complex and intact junction. Tyrosine phosphorylation events by Src or other PTKs can phosphorylate both occludin and ZO-1, disrupting their interactions with each other and reducing junction integrity. DEP-1 is a receptor PTP which is able to dephosphorylate both occludin and ZO-1 and may aid in reforming intact junctions.

Materials and Methods

Cell Culture and Transfections-- MCF10A cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12/Ham medium supplemented with 5% horse serum, 20 ng/ml EGF, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and antibiotics. MDCK II and HEK 293ft cells were cultured in Dulbecco's modified Eagle's medium-high glucose enriched with 10% fetal bovine serum and antibiotics. Cells were transfected with Lipofectamine 2000 (Invitrogen) or Fugene6 (Roche Applied Science) according to the manufacturer's instructions.

Constructs and Antibodies--pMT2 DEP-1 WT and pMT2 DEP-1 D/A (GenBank TM accession number U10886) were kindly provided by Nicholas Tonks (Cold Spring Harbor). pEGFP DEP-1 WT and pEGFP DEP-1 D/A were generated by sub cloning the pMT2 contructs into pEGFP-N2 (Clontech). DEP-1 cytoplasmic domain constructs were generated using the pMT2 DEP-1 wildtype or D1205A point mutant as templates. A 5' primer introduced an EcoR1 before the DEP-1 cytoplasmic sequence at nucleotide 3338 and a 3'primer introduced a Xho1 site after the DEP-1 stop codon. The resulting PCR fragments (nucleotides 3338-4362) were cloned into the EcoR1/Xho1 sites of the pGEX-4T-1 vector (GE Healthcare), creating wildtype and point mutant D1205A pGEX-DEP-1 constructs. The GST-DEP-1 D/A Δ4 construct was generated using the same 5' primer as above and a 3' primer which inserted a stop codon after nucleotide 4350. GST-PTP-PEST wildtype and substrate trapping mutant were obtained from Dr. Sarita Sastry (University of Texas Galveston), GST-PTP μ wildtype and substrate trapping mutant were kind gifts from Dr. Susann Brady-Kalnay (Case Western), and GST SAP-1 constructs were given by Takashi

Matozaki (Gunma University, Japan). Full length occludin was provided by Alan Fanning (University of North Carolina Chapel Hill). Constitutively active (Y527F) src was obtained from Daniel Flynn (West Virginia University). For adenovirus, the full length cDNA of DEP-1 WT was cloned into pENTR1D (Invitrogen*)*. The entry clone was recombined with the pAd/CMV/V5 destination vector via LR clonase II. The ViraPower Adenviral expression vector (Invitrogen Life Technologies) was linearized and then transfected into 293A cells via Lipofectamine 2000 to generate adenovirus encoding V5-tagged DEP-1. The virus particles were used to infect MDCK II cells.

Mouse anti-occludin and rabbit anti-ZO-1 antibodies were purchased from Zymed Laboratories Inc. Mouse anti-human CD148 (DEP-1) antibody was purchased from Biosource. GFP monoclonal antibody was from Roche. Monoclonal antibodies to E-cadherin, moesin and p120ctn were purchased from BD Bioscience, anti-Src was purchased from Millipore, AF-6 rabbit polyclonal was from Novus Biological, and monoclonal PY-20 was purchased from Santa Cruz Biotechnology.

GST fusion proteins-- Expression of the fusion proteins in Escherichia coli were induced with 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside) for 16 hours at room temperature. Bacterial cells were lysed in buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 5 mM MgCl2, 1 mM DTT, 10 μg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and the fusion proteins were purified by incubation with glutathione-sepharose 4B beads (GE healthcare) at 4ºC. Catalytic activity of the fusion proteins was checked by PTP activity assays using pNPP (p-nitrophenylphosphate (Sigma)).

Substrate Trapping Pulldown-- MCF10A cells were either left untreated or treated with 100 μM pervanadate (phosphatase inhibitor) for 15 mins prior to lysis. Cells were rinsed twice in phosphate-buffered saline and lysed in a modified RIPA buffer (1% Tx-100, 0.5% DOC, 0.2% SDS, 150 mM NaCl, 20 mM Hepes ph 7.4, 2 mM EDTA, 10 μg/ml each aprotinin and leupeptin, and 1mM PMSF). Insoluble material was removed by centrifugation. MCF10A lysates (1 mg) were incubated with 10 μg of GST proteins for 1 hour at 4ºC. Beads were washed in the modified RIPA buffer, resuspended in Laemmli sample buffer and analysed by SDS-PAGE and western blotting on PVDF membranes (Millipore).

To determine whether the substrates were binding DEP-1 D/A at the PTP active site, we tested the effect of orthovanadate on complex formation. GST fusion proteins bound to glutathione sepharose were preincubated in a Hepes lysis buffer (1% Tx-100, 150 mM NaCl, 20 mM Hepes, pH 7.5, 5 mM $MgCl_2$,) with or without 2 mM orthovanadate. MCF10A cells were treated with 100 μm pervanadate for 20 mins and also lysed in Hepes lysis buffer with or without 2 mM orthovanadate. Lysates (500 μg – 1mg) were incubated with 2 μg of GST-DEP-1 or GST-DEP-1 D/A for 1.5 hours at 4ºC. Beads were washed in the Hepes Lysis buffer, resuspended in Laemmli sample buffer, and analysed by SDS-PAGE and western blotting on PVDF membranes.

Dephosphorylation Assays-- MCF10A cells were treated with 100 μM pervanadate for 15 mins and lysed in the modified RIPA buffer plus 10 μg/ml each aprotinin and leupeptin, 1mM PMSF, and 5 mM iodoacetic acid (IAA). After incubation of the lysates on ice for 10 mins, DTT was added at a final concentration of 10 mM for another 10 mins on ice to

inactivate the IAA. Lysates were clarified and 500 μg of total cell lysate was incubated with 1 μg glutathione-sepharose bound GST-PTPs for 20 minutes at room temperature. The phosphatases were removed by centrifugation, orthovanadate was added to the samples and occludin was immunoprecipitated for 2 hours at 4 ºC. Beads were washed in the lysis buffer and processed for SDS PAGE. The immune complexes were analyzed by western blotting using the PY-20 antibody, occludin monoclonal antibody or ZO-1 polyclonal antibody. Alternatively, HEK 293ft cells were transfected with pMT2 contructs for 48 hours and lysed directly in hot 2x SDS gel sample buffer (200mM Tris (pH 6.8), 20% glycerol, 4% SDS, 5% 2-ME), and boiled for 10 min. Samples were then diluted 20-fold with phospho-IP buffer (150 mM NaCl, 20 mM Tris, pH 7.6, 1% Triton X-100, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) to a final concentration of 0.1% SDS. One μg of occludin antibody and protein G-sepharose were added and samples were incubated for 4 hours at 4°C. Samples were then washed in TBS and analyzed by Western blot using anti-PY20 antibody.

Immunofluorescence-- MDCK II cells were fixed with 3.7% formaldehyde in phosphate buffered saline and permeabilized in 0.5% Tx-100. Occludin was visualized cells with anti-occludin monoclonal antibody (Zymed) at 1:200 for 45 mins at 37ºC followed by an anti-mouse Alexa-594 conjugated secondary antibody. Images were recorded with a Zeiss LSM510 confocal microscope.

Calcium Switch experiments—MDCK II cells were plated at confluent density (~4 x10^5 cells/cm^2) onto Transwell filters (0.4 μm pore, 12mm diameter; Corning). Growth media was

removed from cells 3-4 hours after plating and replaced with DMEM containing low calcium (5 μM) overnight (~16 hours). Disruption of cell-cell junctions was confirmed by TER measurements of only 2 ohms/cm^2. Junction reassembly was induced with the addition of calcium containing DMEM (1.8 mM $CaCl_2$) for the indicated length of time.

Permeability assays--TER: MDCK II cells were infected with pAd/CMV/GFP or pAd/CMV/V5-DEP-1 WT. 24 hours later, cells were plated at confluent density (~4 $x10^5$ cells/cm^2) onto Transwell filters (0.4 μm pore, 12mm diameter; Corning) and cultured for 4 days, with media replacement daily. TER was measured using an Endohm-12 Transwell chamber connected to an EVOM voltohmeter according to the manufacturer's instructions. For calcium switch experiments, TER was measured at indicated time points following the re-addition of calcium containing media. The resistance of the filter was subtracted from all readings.

FITC-Dextran flux: MDCK II cells were infected with DEP-1 or GFP control adenovirus and plated the same as for the TER measurements above. For the calcium switch, calcium containing DMEM was added to the chambers for indicated length of time; 1 ml in the outer chamber and 250 μl in the inner chamber. 10-kDa FITC-dextran (Molecular Probes) was added to the top chamber in a volume of 50 μl at a final concentration of 1 mg/ml. After 1 hour, a sample was removed from the basolateral (bottom) compartments and read in a fluorometer (FluroStar) (ex 485nm, em 520 nm).

Statistics—Comparison between two groups was made by the Student's *t* test for unpaired data.

.Chapter 3:
Mapping the Region of Occludin Necessary for DEP-1 Interaction

Summary

In the previous chapter novel interactions were discovered between the PTP DEP-1 and members of the tight junction. Specifically, we were able to characterize occludin as a substrate of DEP-1 in epithelial cells. Occludin binds to the catalytic domain of DEP-1 and not to the PDZ tail, however it is not known which region of occludin is responsible for its interaction with DEP-1. Due to the larger number of intracellular tyrosines (23) in occludin, this has been difficult to determine. Using truncation mutants we attempted to map the region in the C-terminal tail of occludin that binds to DEP-1. In pervanadate treated HEK 293ft cells all C-terminal occludin mutants are able to bind the substrate trapping mutant of DEP-1. This suggests that multiple sites may be important for this interaction. Future experiments will focus specifically on residues in the ZO-1 binding site.

Introduction

The importance of occludin in tight junctions remains to be definitively resolved. Mice lacking occludin show no abnormal phenotype and their epithelia still maintained TJ strands and intact barrier function (204). Nevertheless, over-expression studies of full length occludin have demonstrated that occludin is a functional component of the TJ (189, 239). The N-terminal domains of occludin have been implicated in being necessary for correct

localization of occludin. Two studies have reported that occludin lacking the C-terminal cytoplasmic domain can localize to the TJ when endogenous occludin is present (189, 190) suggesting that the N-terminal domain is sufficient for localization. In addition, expression of an N-terminally truncated occludin disrupted tight junction integrity, demonstrating the role of the N-terminal domain of occludin in maintaining epithelial barrier function (193).

In contrast, there is also evidence which supports a necessary role for the C-terminal tail of occludin in correct targeting to the tight junction. Structural and functional data suggest that the C-terminal tail of occludin can be divided into two sub domains. The first 150 amino acids proximal to the membrane (referred to as the proximal domain) are not well conserved among species and are not known to interact with any proteins (196). However, the domain encompassing the last 150 amino acids (also known as the distal domain or the ZO-binding domain) is highly charged and conserved among species. It is this region that binds directly to F-actin, ZO-1, ZO-2, and ZO-3 (197-201). Further, investigators have crystallized the C-terminal tail of occludin and found that amino acids 416-522 are sufficient for ZO-1 binding (240) (Fig. 3.1).

It has been shown that in mutant occludin lacking the extracellular homotypic binding sites, the C-terminal tail of occludin, especially the ZO-binding domain, is necessary and sufficient for its localization to TJs (203). Chimeras of occludin expressing the membrane distal domain of occludin which contains the ZO-binding domain labeled the tight junction in MDCK cells (cells which contain ZO-1). However, the membrane proximal region, which lacks the ZO-binding domain, localized to discrete puncta within the cells (203). These constructs were also expressed in NRK fibroblasts, cells which do not contain occludin or claudins 1 or 2, nor do they generate tight junctions. Again, the ZO-

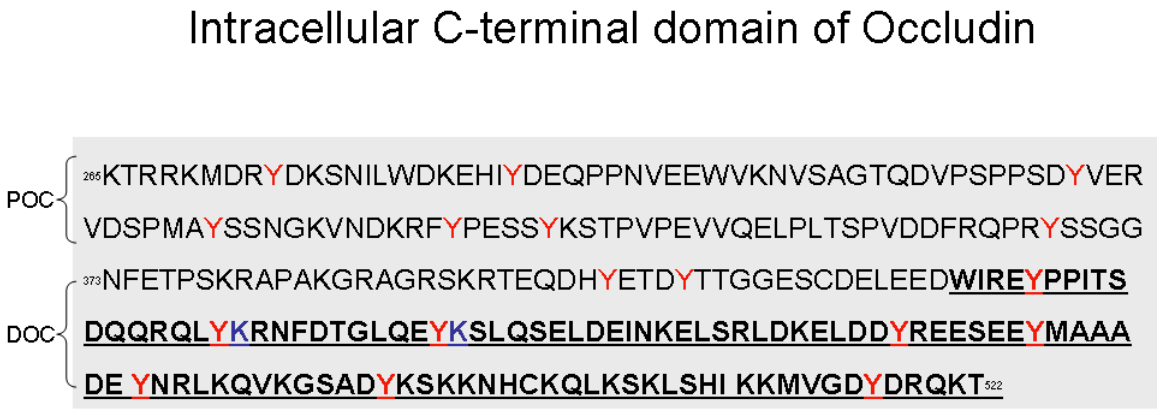

Figure 3.1. The amino acid sequence for the intracellular C-terminal tail of occludin. Proximal domain (POC) consists of amino acids 265-372. Distal domain (DOC) consists of amino acids 373-522. ZO-1 binding domain consists of amino acids 416-522. Tyrosines are in red, critical lysines in blue, and the amino acids sufficient for ZO-1 binding are bolded and underlined.

binding domain of occludin was sufficient to localize occludin to sites containing ZO-1, suggesting that the interactions with ZO proteins contribute to the targeting occludin at the TJ (203).

Stable junctions are believed to exist in a dephosphorylated state and increased tyrosine phosphorylation of both AJ and TJ proteins can affect the stability of these junctions (69, 70, 212). Src is a tyrosine kinase that localizes to the TJ, binds to ZO-1 (232) and can phosphorylate occludin in vitro (217). Phosphorylation of the C-terminal tail of occludin by Src inhibits the binding of ZO-1, ZO-2, and ZO-3 and may be involved in Src-mediated disruption of TJs (217). Therefore, we propose that the distal domain/ZO-binding domain of occludin may be the region which interacts with DEP-1. The entire C-terminal tail of occludin contains 17 tyrosines, with 10 of them in the distal domain (Fig. 3.1). Due to the large number of tyrosines we decided not to mutate individual tyrosines. Instead, we used chimeras and truncation mutants of the cytoplasmic C-terminal tail of occludin to narrow down potential binding domains. We expressed the mutants and conducted pulldown assays with the substrate-trapping mutant of DEP-1 to determine which domain was important for the interaction. We found that tyrosines not only in the distal domain but also the proximal domain of the c-terminal tail are able to interact with DEP-1.

Results

To investigate which domain/tyrosine residue(s) are important for occludin's interaction with DEP-1 we used chimeras of the gap junction protein connexin32 and C-terminal domains of occludin (203). We saw binding of all constructs to GST-DEP-1 D/A, but not to GST with pervanadate treatment (Fig. 3.2). We were concerned that the chimeras

could be binding to DEP-1 via the connexin32 component so we used the GST proteins in a pulldown assay from pervanadate treated MCF10A cells and probed with an anti-connexin32 antibody. Connexin32 is able to interact with the substrate trapping mutant of DEP-1 (data not shown) and thus we could not conclude whether the chimeras were binding due to interactions with connexin32 or occludin.

With this knowledge, we developed truncation mutants of human occludin that were similar to those used by Mitic et al (203), but without the connexin32 transmembrane domains. First, we generated these mutants in pEGFP-N2 but found the GFP from pervanadate-treated cells non-specifically interacted with the substrate-trapping mutant of DEP-1, again confusing our results (data not shown). As a result, we generated similar myc-tagged constructs (Fig. 3.3). The myc tag does not contain a tyrosine and should not be phosphorylated by pervanadate treatment. HEK 293ft cells were transfected with full length occludin as well as the mutants. 48-hrs post transfection cells were treated with pervanadate and lysates were used in a substrate trapping mutant pulldown. Full length occludin, as well as all three mutants, bound to DEP-1 D/A (Fig. 3.4).

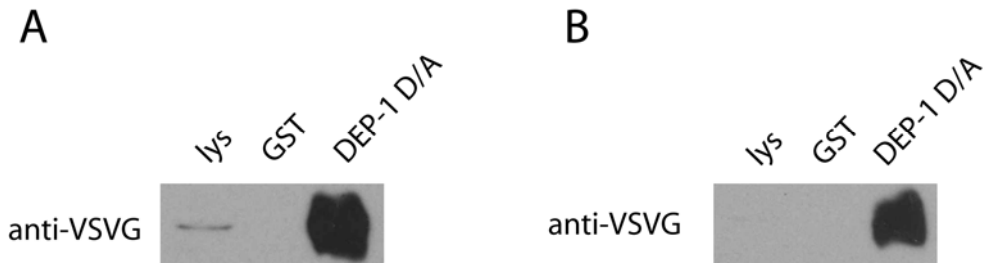

Figure 3.2. Connexin32 Chimeras of occludin interact with DEP-1 D/A. The VSVG-connexin32 chimeras (A) DOC and (B) POC were transfected into HEK 293ft cells and 48 hours later they were treated with 100 μm pervanadate for 15 mins and lysed. Lysates were used in a pulldown assay with GST or GST DEP-1 D/A, resolved by SDS-PAGE and membranes were probed with an anti-VSVG antibody. *Although no POC appears in the lysate of B, a longer exposure confirmed that the protein is in fact expressed in the cells.

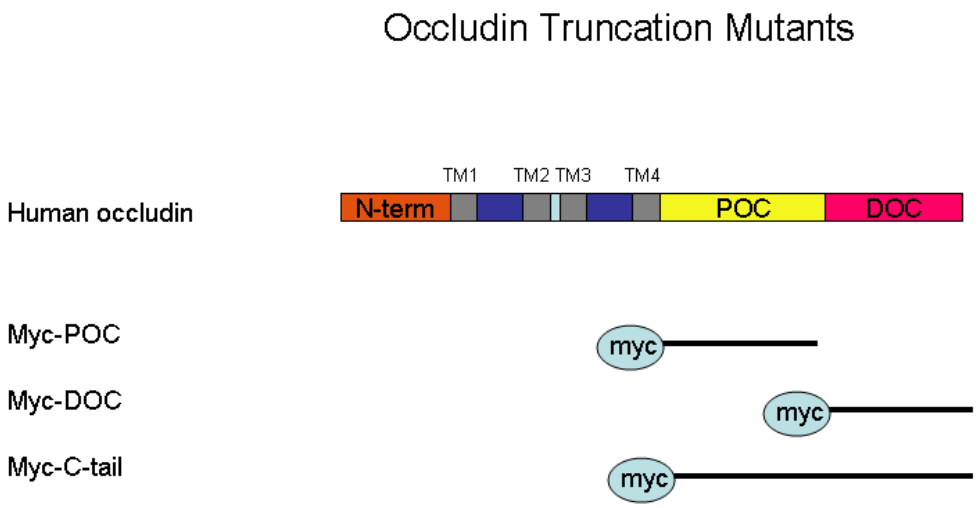

Figure 3.3. Schematic of Myc-tagged Truncation Mutants of Occludin. Full length human occludin is depicted at the tope. The transmembrane regions (TM) are gray with the extracellular loops dark blue and the small intracellular loop in light blue. All truncation constructs are N-terminally tagged with myc. The C-tail mutant contains both the POC and DOC.

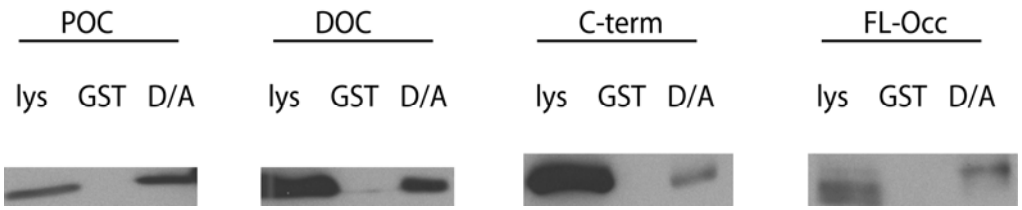

Figure 3.4. Truncation mutants of occludin interact with DEP-1 D/A. HEK 293 ft cells were transfected with myc-tagged occludin truncation mutants. Cells were treated with 100 μm pervanadate for 15 mins and then lysed. 1 mg of cell lysate was incubated with GST or GST DEP-1 D/A and samples were resolved with SDS-PAGE and western blotting with a 9E10 myc monoclonal antibody or occludin antibody (for FL-Occ).

Discussion

This study examined whether an interaction between DEP-1 and occludin occurred by using truncation mutants of the occludin C-terminal tail. We show that pervanadate treatment induced binding of all the occludin cytoplasmic truncations to DEP-1. Based on the literature, we had hypothesized that the distal domain of occludin containing the ZO binding domain would be the most crucial (203). However, in our experiments, the proximal domain was also able to interact with DEP-1 following pervanadate treatment. One caveat to these experiments is that we have not examined binding of these mutants in cells not treated with pervanadate. Previous work (chapter 2) has never demonstrated an interaction in untreated cells but we will examine this in the future. In addition, we have not directly shown that pervanadate treatment induced phosphorylation of these mutants. Since pervanadate treatment has phosphorylated full length occludin in previous experiments, it is expected to do so with these mutants but this will be tested. The co-expression of constitutively active Src and the myc-tagged POC and DOC mutants in HEK 293ft cells induced phosphorylation of the mutants, demonstrating they are capable of being phosphorylated (data not shown).

With 17 tyrosines in the C-terminal tail, the possibility exists that DEP-1 may bind to multiple phosphorylation sites. One major experimental factor that needs to be considered is the use of pervanadate to induce phosphorylation. Pervanadate inhibits all tyrosine phosphatases and so causes a global increase in intracellular tyrosine phosphorylation levels. This treatment may cause the phosphorylation of certain tyrosines that under "normal" conditions are not phosphorylated, and result in false positive interactions. A better way to better determine binding sites would be to induce phosphorylation with a known kinase, such as Src, that phosphorylates the C-terminal tail of occludin (217). There are several Src

kinase recognition motifs in both the proximal domain as well as the distal domain which may be potential DEP-1 binding sites. To test this, constitutively active Src could be co-transfected with the truncation mutants and used in DEP-1 substrate-trapping mutant pulldowns to determine which, if any, Src phosphorylated tyrosines can bind to DEP-1.

There are also several EGFR recognition motifs in the cytoplasmic tail of occludin, suggesting that the addition of EGF may stimulate this pathway and result in site-specific phosphorylation. Preliminary experiments have been done examining EGF-induced phosphorylation of occludin; unfortunately, it has been difficult to show occludin phosphorylation specifically. While generalized tyrosine phosphorylation of lysates increases with EGF treatment, it may require optimization of immunoprecipiation conditions in order to determine specific occludin phosphorylation. In addition, EGFR is a substrate of Src and can be phosphorylated on Y845, an autophosphorylation site (241, 242). As a result, Src may also be able to induce the phosphorylation of EGFR motifs indirectly. One would have to keep this in mind when evaluating interaction data. Regardless, these two kinases would more specifically phosphorylate the C-terminal tail compared to treatment with pervanadate and future studies will examine their effect on occludin-DEP-1 interactions.

The C-terminal distal domain of occludin has been crystallized and from this study it was determine that amino acids 416-522 contain the binding site for ZO-1 and are important for occludin localization to cell-cell junctions.(240). The surface charge profile found a large midsection of the distal domain which is highly basic and conserved among species. Double point mutations which changed lysine pairs to aspartic acid and reversed the charge of this section prevented the binding of ZO-1 to occludin (Lys433/511, Lys444/504, and Lys 485/488). A single point mutation at Lys 433 was also able to disrupt binding of ZO-1. This

data suggested that the positive charged surface created by the lysine residues on the alpha helices of the distal domain of occludin created a binding site for the GUK domain of ZO-1 (240). Interestingly, both Lys 433 and 444 have a tyrosine residue adjacent to them (Fig. 3.1). We propose that because phosphorylation of one or both of these tyrosines will result in a negative charge in nearly the same location as the Lys->Asp mutation, phosphorylation might similarly disrupt occludin binding to ZO-1. Therefore, future work should include mutating these two tyrosines to the phosphomimetic glutamic acid and determining if interaction with ZO-1 still occurs. If these mutations successfully inhibit ZO-1 binding it suggests that tyrosine phosphorylation of these residues may be sufficient to attenuate the occludin and ZO-1 interaction. In addition, mutations of Tyr432 and Tyr443 to phenylalanine will be used to determine if the occludin-DEP-1 interaction is dependent on phosphorylation of these residues.

It has been shown previously that DEP-1 can selectively dephosphorylate several growth factor receptors in a site selective manner to control signaling (149, 152, 153, 226). It is possible that DEP-1 can bind and dephosphorylate multiple tyrosines on the C-terminal tail of occludin, which could modulate protein-protein interactions (such as ZO-1), controlling tight junction protein complex formation and thus the barrier function of tight junctions. The site selectivity that DEP-1 displayed in PDGF β-receptor dephosphorylation was predominantly determined by the primary amino acid sequence surrounding the phosphotyrosine. Poorly dephosphorylated tyrosines have basic residues at positions -4 and +3 relative to phosphotyrosine (153, 226). Lysine at position +3 seems to be the most crucial with regards to low affinity for the phosphotyrosine, with a lysine at +1 being better accepted. For that reason, Tyr432 and Tyr443 are still acceptable potential

dephosphorylation sites for DEP-1 on occludin. Tyr443 might be the better of the two residues since it contains a leucine at +3 and a glycine at -4, both non-polar hydrophobic residues which have been shown to be favored by DEP-1 (226). It is also possible that the susceptibility of dephosphorylation of a residue may be affected by the phosphorylation status of neighboring tyrosine residues. This again emphasizes the role of site specific phosphorylation by kinases and upstream signaling pathways in deciding which PTP acts on a substrate at any given time.

Our results suggest that several tyrosines in the C-terminal tail of occludin may be phosphorylated and consequently bound by the substrate trapping mutant of DEP-1. Further work needs to examine DEP-1 binding after phosphorylation by specific kinase activity to avoid excessive, non-physiologic effects of pervanadate. In addition, based on the identification of residues necessary for ZO-1 binding, specific tyrosines adjacent to critical lysines in the ZO-1 binding region will also be examined via point mutations studies. Mapping the residues necessary for DEP-1 interaction will help to clarify potential signaling pathways and kinases that DEP-1 activity may be counteracting. In addition, we may determine additional kinases that may be involved in phosphorylation of occludin.

Materials and Methods

Constructs and Antibodies- VSVG-tagged full length human occludin and connexin 32-occludin chimeras were obtained from Alan Fanning (University of North Carolina Chapel Hill) and are described previously (194, 203). Myc-tagged truncations of the C-terminal tail of occludin were made similar to Mitic et al. (203). The proximal C-terminal residues 265-372 of occludin (POC), the distal c-terminal residues (373-522) (DOC) as well as the entire

C-terminal tail (265-522) (C-tail) were first inserted into pEGFP-N2 by amplifying this region and digesting with BglII and HindIII.. These constructs were then digested with BglII and KpnI and inserted into pCMV-Myc-J3 (modified Clontech vector from Jia Zhong at UNC-CH). VSVG monoclonal antibody is from x. 9E10 myc monoclonal was supernatant produced in the Burridge Lab.

Cell Culture and Transfections- HEK 293ft cells were cultured in Dulbecco's modified Eagle's medium-high glucose enriched with 10% fetal bovine serum and antibiotics. Cells were transfected with Fugene6 (Roche Applied Science) according to the manufacturer's instructions.

GST fusion proteins and Substrate Trapping Pulldown—GST and GST-DEP-1 D/A were prepared as described in Chapter 2 material and methods. HEK 293ft cells were transfected with myc-tagged occludin truncations or full length occludin and processed 48 hours later. Cells were treated with 100 μM pervanadate for 15 mins to induce phosphorylation prior to lysis. Cells were rinsed twice in phosphate-buffered saline and lysed in a modified RIPA buffer (1% Tx-100, 0.5% DOC, 0.2% SDS, 150 mM NaCl, 20 mM Hepes ph 7.4, 2 mM EDTA, 10 μg/ml each aprotinin and leupeptin, and 1mM PMSF). Insoluble material was removed by centrifugation. HEK 293ft lysates (1 mg) were incubated with 10 μg of GST or GST DEP-1 D/A for 1 hour at 4ºC. Beads were washed in the modified RIPA buffer, resuspended in Laemmli sample buffer and analysed by SDS-PAGE and western blotting on PVDF membranes (Millipore).

Chapter 4:

Focal adhesion proteins FAK and paxillin are substrates of DEP-1

Summary

FAK and paxillin localize to focal adhesions, points of adhesion between a cell and the extracellular matrix (ECM). In response to adhesion to ECM proteins such as fibronectin, both proteins become tyrosine phosphorylated. This tyrosine phosphorylation affects their activity and recruits binding partners. Consequently, they are potential substrates for PTPs. In this study, FAK and paxillin bound to the substrate trapping mutant of DEP-1, and were found to be dephosphorylated by wild type DEP-1. These data suggest that FAK and paxillin are substrates of DEP-1. However, how DEP-1 regulates these proteins and their downstream signaling pathways is unknown. Therefore, future studies will need to address this issue.

Introduction

Post translational modifications such as tyrosine phosphorylation are critical to several signaling pathways downstream from cell-cell and cell-matrix adhesion. Phosphotyrosine levels are regulated by the balance between PTK and PTP activity. Although a number of kinases are known to be involved in increasing phosphorylation at sites of cell-matrix contact, the PTPs which act there are less understood. In the previous chapters, substrates and signaling pathways involving DEP-1 and cell-cell adhesions were

discussed. Here we examine DEP-1's role in regulating the phosphorylation of proteins associated with cell-matrix adhesions.

Focal adhesions are sites of strong adhesion to the ECM in cells grown in tissue culture. They anchor stress fibers to the plasma membrane via integrins and are multiprotein signaling complexes for a large number of signaling pathways downstream of integrin mediated adhesions (60). Both Src and focal adhesion kinase (FAK) are known to localize here and phosphorylate several of the focal adhesion proteins (4). In addition, paxillin is a focal adhesion protein that is phosphorylated by several stimuli including integrin dependent cell adhesion and the tyrosine kinase activity of FAK and CAKβ (243-245). Paxillin is a signaling molecule that plays an important role as an adaptor protein, recruiting cytoskeletal and signaling proteins to focal adhesions. FAK and paxillin are known substrates for the phosphatases Shp-2, PTP-PEST and SAP-1 (18, 31, 236). To test whether FAK and paxillin might also be substrates for DEP-1 we used the substrate trapping mutant of DEP-1 (described in Chapter 2). We found that they were in fact able to bind the DEP-1 D/A. In addition, wild type DEP-1 was able to dephosphorylate the proteins suggesting FAK and paxillin are substrates of DEP-1. We propose that DEP-1 is not only able to act on junctional proteins but may play a role in the regulation of cell-matrix adhesions as well as adhesion-mediated signaling pathways.

Results

FAK and paxillin bind to the DEP-1 substrate trapping mutant

We examined whether other known tyrosine phosphorylated proteins important in cell-matrix interactions are substrates of DEP-1. We treated MCF10A cells with

pervanadate, a global PTP inhibitor, and used the lysate in a DEP-1 substrate trapping mutant pulldown. Both FAK and paxillin were able to bind to DEP-1 D/A in a tyrosine phosphorylation dependent manner (Fig 4.1A), as well as a dose dependent manner (Fig. 4.1B). We wanted to determine if the interaction of DEP-1 D/A with FAK and paxillin was specific or if these proteins were substrates of additional PTPs. Lysates of untreated or pervanadate treated MCF10A cells were incubated with GST fusion proteins of the substrate trapping mutants of DEP-1 (GST-DEP-1 D/A), PTP-PEST (GST-PTP-PEST D/A), and Shp2 (GST-Shp2 C/S). With a short exposure, DEP-1 D/A was the only PTP of those tested that was able to interact with FAK and paxillin and as observed previously (Fig. 4.1A) it did so in a tyrosine phosphorylation-dependent manner (Fig. 4.2A). A longer exposure showed weak binding of paxillin to PTP-PEST (Fig. 4.2B). Previous studies have shown that paxillin is a substrate of PTP-PEST (18ref, 246), although interaction with the substrate trapping mutant (PEST D/A) has not previously been demonstrated.

DEP-1 can dephosphorylate FAK and paxillin in vitro

DEP-1 D/A can bind to both FAK and paxillin as determined by GST fusion protein pulldowns (Fig. 4.1 and 4.2). We wanted to determine if FAK and paxillin were substrates for wild type DEP-1 as well as other wild type PTPs *in vitro*. MCF10A cells were treated with pervanadate prior to lysis. FAK and paxillin were immunoprecipated from the lysates and then incubated with purified GST-PTPs. Phosphotyrosine levels of FAK and paxillin were analyzed by immunoblotting with anti-phosphotyrosine antibodies. Wildtype DEP-1 was able to dephosphorylate FAK and paxillin; where as other PTPs tested had minimal effects (Fig. 4.3A and 4.3B). All PTPs used were tested for catalytic activity using the substrate pNPP.

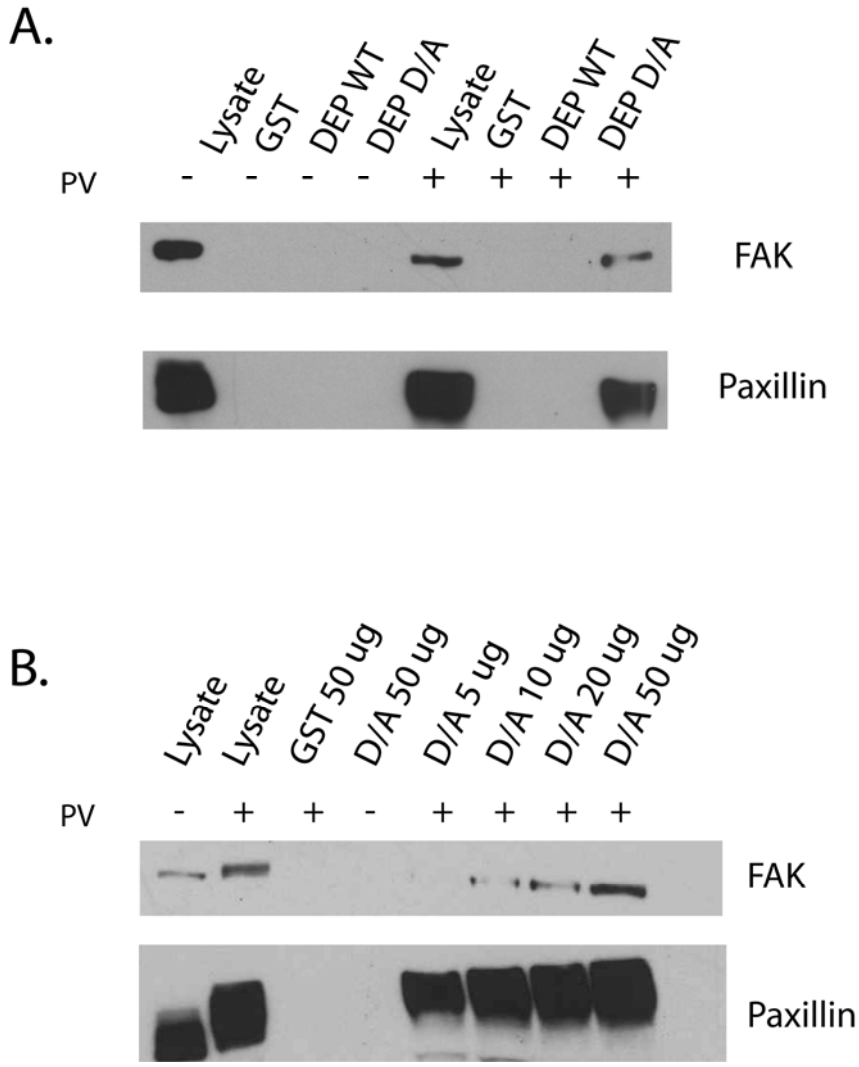

Figure 4.1. FAK and Paxillin interact with the substrate trapping mutant of DEP-1. **A**. . MCF10A cells were either left untreated (-) or were treated (+) with 100 μM pervanadate (PV) for 10 mins prior to lysis. GST alone, GST-DEP-1 WT or GST-DEP-1 D/A fusion proteins (10 μg) were incubated with cell lysates and protein complexes were analyzed by SDS-PAGE and immunoblotting with FAK or paxillin antibodies. **B**. FAK and paxillin bind DEP-1 D/A in concentration dependent manner. MCF10A cells were treated as described for A. Lysates were incubated with the indicated amounts of GST proteins and protein complexes were analyzed by SDS-PAGE and immunoblotting with antibodies to FAK and paxillin.

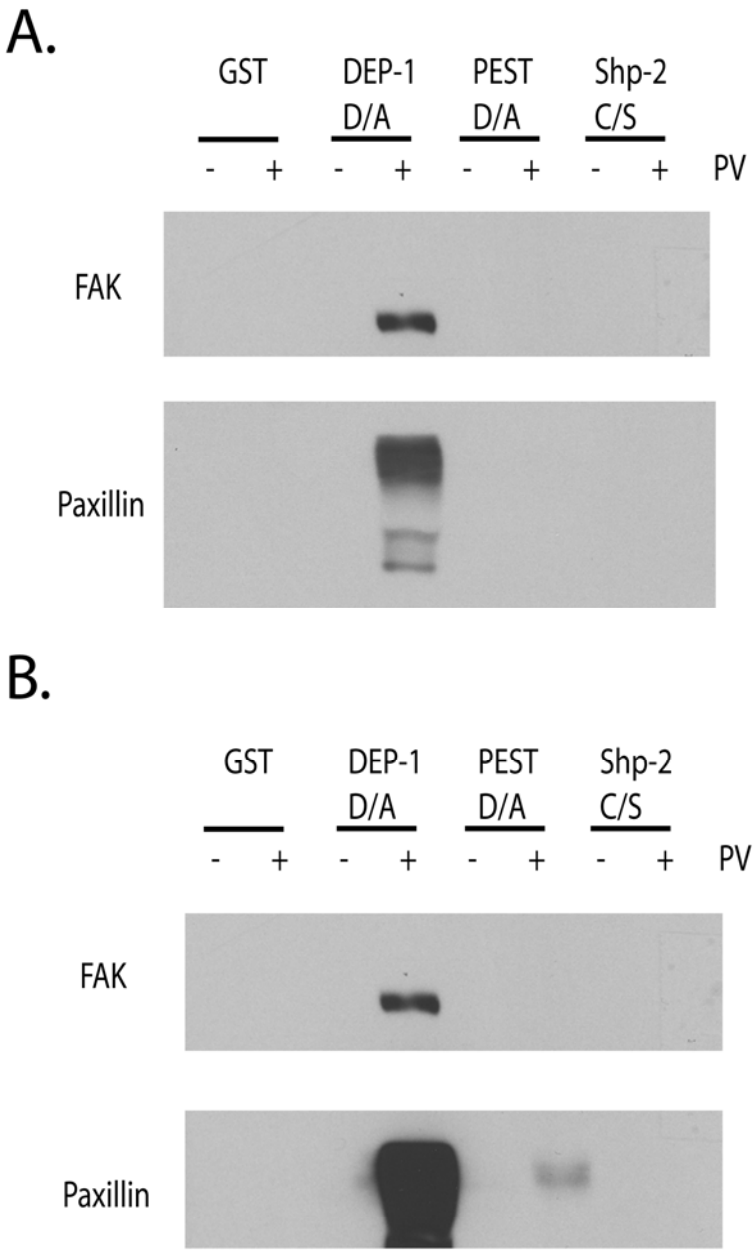

Figure 4.2. FAK and Paxillin interact with DEP-1 with a higher affinity than other PTP trapping mutants. MCF10A cells were either left untreated (-) or were treated (+) with 100 μM pervanadate (PV) for 10 mins prior to lysis. Lysates were were incubated with GST or the GST-fusion proteins of the substrate trapping mutants of DEP-1, PTP-PEST or Shp2. Protein complexes were analyzed by SDS-PAGE and immunoblotted for FAK and paxillin. **A**. Short time exposure shows DEP-1 D/A interacting with FAK and paxillin. **B**. Longer exposure confirms binding of paxillin, a known substrate, to PTP-PEST, but to a much lesser extent than DEP-1.

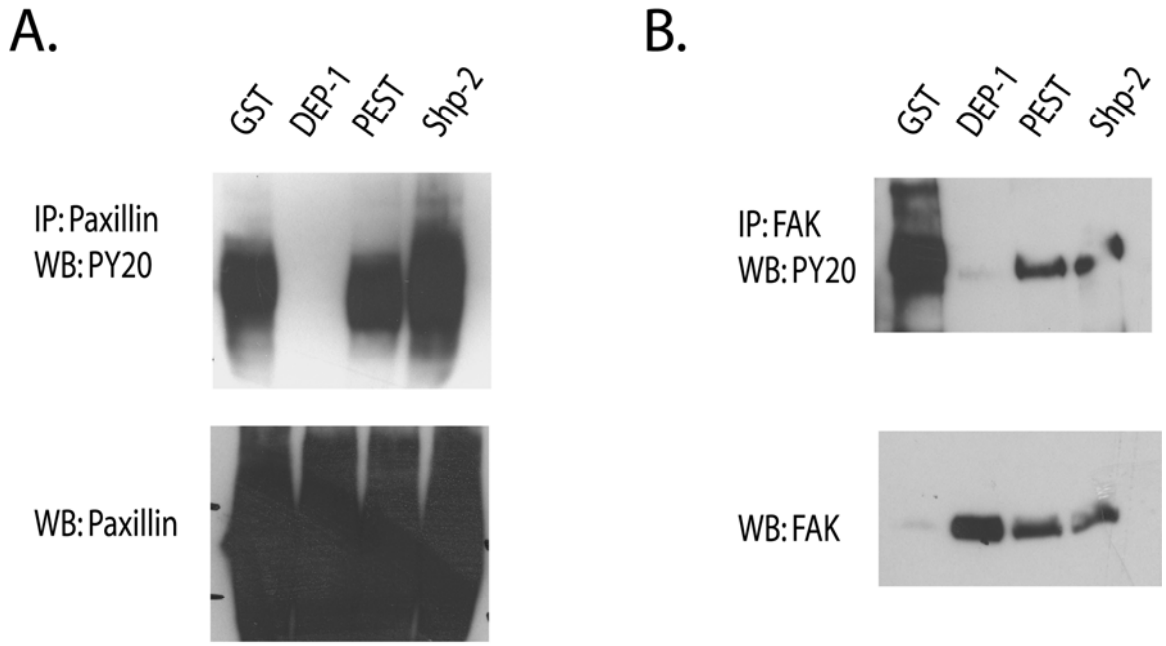

Figure 4.3. DEP-1 dephosphorylates FAK and paxillin *in vitro*. MCF10A cells were treated with 100 μM pervanadate for 10 mins prior to lysis. **A**. Paxillin and **B.** FAK were immunoprecipitated from lysates. Immunocomplexes were then incubated with GST or wildtype GST-PTP fusion proteins bound to glutathione sepharose for 30 mins at 65ºC. Immunocomplexes were analyzed by SDS-PAGE and immunblotted with a PY-20 antibody. Membranes were then stripped and reprobed with the immunoprecipitated antibody (**A**. paxillin, **B**. FAK) to ensure equalized protein was immunoprecipitated.

Discussion

Here we have identified FAK and paxillin as two novel substrates for the receptor PTP DEP-1. DEP-1 is a single pass transmembrane PTP which localizes to the plasma membrane and is enhanced at points of cell-cell adhesion. DEP-1 has not been shown to directly localize at focal adhesions, but neither has PTP-PEST (247), a PTP known to dephosphorylate paxillin. DEP-1 might be associated with a population of paxillin and/or FAK not located at focal adhesions. Also, two PTPs related to DEP-1, SAP-1 and GLEPP1, negatively regulate intergrin-mediated signaling by dephosphorylating p130Cas and disrupting focal contacts and cytoskeletal components (236, 248). Therefore, DEP-1's expression at the plasma membrane as well as its homology to other PTPs with known focal adhesion substrates suggests that DEP-1 may interact with FAK and paxillin proteins *in vivo*.

DEP-1 may be both a positive and negative regulator of adhesion-mediated signaling. DEP-1 binds c-Src and dephosphorylates the inhibitory tyrosine 529, thereby increasing Src kinase activity (172). Adhesion, along with tyrosine phosphorylation of both FAK and paxillin, were increased in these cells compared to untransfected or cells expressing the catalytic inactive mutant (DEP-1 C/S). Inhibition of c-Src activity with PP2 decreased these responses, suggesting DEP-1 may be positive regulator of Src acvitiy in the context of integrin-mediated adhesion and may increase levels of phosphotyrosine in FAK and paxillin (172). Additional data have also confirmed that over expression of DEP-1 increases cell adhesion (157), strengthening this model.

However, DEP-1 may also negatively affect adhesion-mediated signaling cascades. When DEP-1 was over-expressed in NIH 3T3 fibroblasts there was a reduction in association of Src with PDGFR-β concomitant with a decrease in FAK phosphorylation (92).

Furthermore, 48 hours after induction of DEP-1 expression, virtually no paxillin containing focal adhesions were seen in the fibroblasts, however, the control cells appeared normal. DEP-1 cells were able to make adhesions with the extracellular matrix protein fibronectin, however, the cells had smaller, dot like peripheral structures which were short lived and failed to induce phosphorylation of FAK compared to control cells. These focal complexes were able to form, but did not mature into stable focal adhesions like control cells. Moreover, DEP-1 cells exhibited decreased migration in response to PDGF and were much smaller and more rounded demonstrating both a cell spreading and cell migration defect (92). Therefore, several signaling pathways may be controlled by DEP-1. DEP-1 may be regulating phosphorylation of FAK indirectly by dephosphorylating PDGFR-β, thereby attenuating the PDGFR-β-Src interaction, and modulating Src activity and other downstream signaling pathways. Also, DEP-1 over expression might be inhibiting Rho GTPase activity. Previous studies found PTP-PEST decreased Rac1 activity and inhibited cell migration by controlling the phosphorylation levels of GEFs and GAPs (10, 11). Several GEFs and GAPs for the Rho family are regulated by tyrosine phosphorylation, including the Rac GEF VAV2 and the Rho GAP p190RhoGAP (249, 250). These proteins may be substrates for DEP-1 as well. Interestingly, our data provides evidence that DEP-1 could also directly be dephosphorylating FAK and paxillin. In this way, DEP-1 could either prevent focal adhesion assembly or disassembly by regulating tyrosine phosphorylation of specific residues in FAK. Modulation of paxillin phosphorylation levels may alter its interactions with downstream effectors and affect signaling pathways. There are several possible mechanisms and signaling pathways by which DEP-1 could be exerting an effect on phosphorylation levels of

FAK and paxillin. Further studies will need to be conducted to determine if these mechanisms are influenced by cell type, matrix, or additional stimuli.

Paxillin is a known substrate for PTP-PEST (18, 246). We found that the substrate trapping mutant of DEP-1 was able to bind to paxillin and wild type DEP-1 can dephosphorylate it, suggesting paxillin is also a substrate of DEP-1. Although paxillin may be a substrate for both PTPs, the mechanisms by which the protein-protein interactions occur between paxillin and the PTPs might be different. PTP-PEST binds paxillin via its C-terminal non-catalytic domain and paxillin's C-terminal LIM domains (18, 246, 251). Therefore, the non catalytic domain interaction recruits paxillin to PTP-PEST and facilitates paxillin's dephosphorylation (246). Previous work was unable to show an interaction between the substrate trapping mutant (C/S) of PTP-PEST with paxillin (251). However, the substrate trapping mutant (D/A) of PTP-PEST was able to weakly interact with paxillin in our study. The C/S mutant is known to be a less efficient trapping mutant (8) and so it could be a combination of the different mutation and a larger amount of GST fusion protein used in the pulldowns that enabled us to see this interaction. Although we are seeing a small amount of paxillin binding to GST-PTP-PEST D/A, the primary interaction between these two proteins is driven independently of the PTP-PEST catalytic domain. On the other hand, DEP-1 D/A showed a very robust interaction with paxillin even when low levels of GST-DEP-1 were used (Fig. 4.1), suggesting paxillin may be interacting directly with the catalytic domain of DEP-1 and not through an accessory binding event. Testing the ability of PTP inhibitor orthovanadate to block the interaction between DEP-1 and paxillin will help to determine whether paxillin is exclusively interacting via DEP-1's catalytic domain.

FAK has previously been shown to be a poor substrate for PTP-PEST (41). We have confirmed this data, demonstrating that FAK binds neither the PTP-PEST trapping mutant, nor is it dephosphorylated by wild type PTP-PEST *in vitro* (Fig. 4.2A and 4.3B). However, FAK is a very strong candidate substrate for DEP-1. FAK not only interacts with the substrate trapping mutant of DEP-1 after pervanadate treatment, but FAK is dephosphorylated by recombinant wild type DEP-1 *in vitro* (Fig. 4.1 and 4.3B). In addition to the possibility that DEP-1 acts on FAK at focal adhesions, recent work has placed FAK at cell-cell junctions. FAK has been shown to be important in junction as they form. For example, when Hela cells were depleted of FAK, N-cadherin mediated cell-cell contacts were inhibited in subconfluent cultures (252). Expression of constitutively active FAK in NBT II cells resulted in no change in junctions; however, expression of inactive FAK drastically diminished cell-cell adhesions at early time points following a calcium switch (253). During junctional reassembly, active FAK could be seen at junctions in only approximately 10% of cells and it was never seen in already confluent cultures, suggesting FAK may only transiently associate at junctions (253). Therefore, FAK may be involved in the formation and possible stabilization of cell-cell adhesions. It will be interesting to test if DEP-1 may regulate phosphorylation of Tyr 397 and influence junction formation.

Additionally, identifying other potential tyrosine residues dephosphorylated by DEP-1 would aid in the delineation of the role of DEP-1 in paxillin and FAK related focal adhesion and cell-cell junction signaling. There are numerous phospho-specific antibodies available for FAK and paxillin and it would be very informative to perform DEP-1 substrate trapping mutant pulldowns to determine which residue(s) is binding to the PTP. Also, DEP-1 could be overexpressed or reduced with siRNA in cells and phosphorylation changes could

be examined. To further clarify signaling pathways, phosphorylation could be assessed following various stimuli. For example, following a calcium switch, phosphorylation levels of several tyrosine residues in FAK were examined. Little or no change was found with tyrosines 397, 576, 577, or 925. However, two hours after calcium replenishment Tyr 861 displayed elevated phosphorylation (253). This increase in phosphorylation was dependent on the interaction with E-cadherin. These findings make tyrosine 861 an attractive candidate residue DEP-1 may regulate with regards to cell-cell contact formation. Another interesting residue to examine is Tyr 925. As described in Chapter 1, phosphorylation of Tyr 925 in FAK is necessary to induce an interaction with dynamin at focal adhesions, a critical step in focal adhesion disassembly (62). It would be interesting to examine this residue and focal adhesion disassembly in DEP-1 knockdown cells.

Although we see complete dephosphorylation of FAK and paxillin when incubated with wildtype DEP-1 it is possible that the pervanadate treatment only phosphorylated certain tyrosines. Also, DEP-1 may preferentially dephosphorylate certain residues *in vivo*, but the use of high levels in the *in vitro* assay may result in total dephosphorylation. The experiments presented here were conducted with confluent epithelial cells. These cells do not contain a large amount of focal adhesions, if any. Future experiments will examine DEP-1 substrates in fibroblast cell lines.

There is the possibility that there might be cross talk between cell-cell junctions and focal adhesions. High RhoA is disruptive to AJ formation (110-112). As junctions from, Rac1 activity is increased and RhoA activity is decreased as direct response to cadherin-cadherin engagement (115). Cadherin engagement induces src-dependent tyrosine phosphorylation of p190RhoGAP, thus leading to the observed decrease in RhoA (116).

Epithelial cells need low levels of RhoA to reduce their intracellular tension and contractility and promote the formation of cell-cell adhesions (254). Low RhoA levels also turn off focal adhesion signaling. DEP-1 may be involved in regulating both cell-cell junction and focal adhesion signaling at the same time.

Methods and Materials

Cell Culture-- MCF10A cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12/Ham medium supplemented with 5% horse serum, 20 ng/ml EGF, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and antibiotics.

Constructs and Antibodies—GST DEP-1 constructs were generated as described in Chapter 2. GST PTP-PEST constructs were kindly provided by Sarita Sastry (University of Texas Galveston). The monoclonal PY-20 was purchased from Santa Cruz Biotechnology. Monoclonal FAK and paxillin antibodies were from BD Transduction labs.

GST fusion proteins-- Expression of the fusion proteins in Escherichia coli were performed as previously described in Chapter 2.

Substrate Trapping Pulldown-- MCF10A cells were either left untreated or treated with 100 μM pervanadate (phosphatase inhibitor) for 15 mins prior to lysis. Cells were rinsed twice in phosphate-buffered saline and lysed in a modified RIPA buffer (1% Tx-100, 0.5% DOC, 0.2% SDS, 150 mM NaCl, 20 mM Hepes ph 7.4, 2 mM EDTA, 10 μg/ml each aprotinin and leupeptin, and 1mM PMSF). Insoluble material was removed by centrifugation. MCF10A

lysates (1 mg) were incubated with 10 μg of GST proteins (Figure 4.1) or 100 μg GST proteins (Figure 4.2) for 1 hour at 4ºC. Beads were washed in the modified RIPA buffer, resuspended in Laemmli sample buffer and analysed by SDS-PAGE and western blotting on PVDF membranes (Millipore).

Dephosphorylation Assays-- MCF10A cells were treated with 100 μM pervanadate for 15 mins and lysed in the modified RIPA buffer plus 10 μg/ml each aprotinin and leupeptin, 1mM PMSF, and 5 mM iodoacetic acid (IAA). After incubation of the lysates on ice for 10 mins, DTT was added at a final concentration of 10 mM for another 10 mins on ice to inactivate the IAA. Lysates were clarified and FAK or paxillin was immunoprecipitated from 1 mg lysate with 2 μg antibody for 2 hours at 4ºC. IPs were washed, resupsend in a succinate buffer (50 mM succinate pH 6.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) and incubated with 1 μg glutathione-sepharose bound GST-PTPs for 30 minutes at 30ºC. The reaction was quenched washing 1 time in the succinate buffer and resuspending in Laemmli sample buffer. The immune complexes were run on SDS-PAGE gels and analyzed by western blotting using the PY-20 antibody, FAK monoclonal antibody or paxillin monoclonal antibody.

Chapter 5:

Conclusions and Future Directions

A key element regulating cell-cell and cell-matrix adhesion is tyrosine phosphorylation of proteins at intercellular junctions and focal adhesions. In this dissertation, DEP-1 has been shown to bind to and dephosphorylate proteins located at both locations. In chapter 2, occludin and ZO-1 were identified as novel substrates for the RPTP DEP-1. These proteins appear to be substrates specific for DEP-1 and potentially a related PTP, SAP-1. DEP-1 and SAP-1 are extremely similar to each other, each with 8 fibronectin type III repeats and a single catalytic domain (89, 230). Therefore, occludin and ZO-1 might be substrates specific to this particular family of PTPs. It would be interesting to see if VE-PTP is also able to act on these TJ proteins. We also provided evidence that the over-expression of DEP-1 increased transepithelial electrical resistance in confluent monolayers and reduced paracellular flux of FITC-dextran following a calcium switch. Through dephosphorylation of junctional components, DEP-1 may enhance the stability of protein complexes involved with maintaining junctional integrity. Future work will focus on identifying where DEP-1 interacts with occludin (Chapter 3). This would enhance our understanding of the specificity of DEP-1's catalytic domain, including its recognition of the amino acid sequence surrounding the phospho-tyrosine. It is possible that there are multiple binding and dephosphorylation sites on occludin, depending on upstream kinase activity. We propose that Src kinase phosphorylates occludin and disrupts its interaction with DEP-1, but

would also like to test growth factor receptors such as EGFR. In addition, Chapter 4 discussed the identification of FAK and paxillin as potential substrates for DEP-1. Phosphorylation of both FAK and paxillin occurs downstream from integrin-mediated signaling and DEP-1 may therefore be involved with these pathways as well. Also, phosphorylation of FAK occurs at cell-cell junctions during junction reassembly (253) and may be another way in which DEP-1 may control cell-cell adhesion. Experiments still need to be conducted in order to determine exactly how DEP-1 is controlling physiological processes associated with dephosphorylation of these focal adhesion proteins.

The work presented here has demonstrated a role for the RPTP in controlling cell adhesions in epithelial cells. The following chapter will discuss directions for future research regarding DEP-1 and other RPTPs. First, we will focus on the possibility that RPTPs are involved in the process of leukocyte transmigration. Next we will examine the importance of the extracellular domain in regulating PTP activity. Finally, we will end with a look at PTPs in cancer and the development of PTP inhibitors.

DEP-1 as a regulator of leukocyte transmigration

The vascular endothelium controls the passage of macromolecules and immune cells across it. There are two known mechanisms for leukocyte migration across the endothelium: transcellular and paracellular. Direct evidence supports the existence of transcellular migration (i.e. through a cell), although it is believed to occur in only 5-10% of transmigratory events *in-vitro* (255). Thus, the predominant route for leukocyte transmigration is paracellular migration, where leukocytes migrate between adjacent endothelial cells through their junctions (256). In the resting state, the endothelium

maintains its barrier properties and prevents the passage of cells out of the bloodstream and into tissues. When an inflammatory response is elicited, however, the properties of the endothelium change and leukocytes are able to adhere to and migrate through gaps at the intercellular junctions. The final step in the adhesion cascade of leukocyte transmigration is diapedesis, or the crawling of the leukocyte between adjacent endothelial cells. In order for this to occur, the leukocyte must breach a physical barrier of junctional structures including TJs and AJs (for review see (257)). It has been shown that the adhesive strength of the TJ and AJ is regulated by the tyrosine phosphorylation levels of their protein components. An increase in tyrosine phosphorylation dissociates the junctional complexes from the actin cytoskeleton and reduces junctional integrity (73, 74, 214, 217). Therefore, regulation of tyrosine phosphorylation levels of junctional components is an important mechanism for regulating both the stability and permeability of tight and adherens junctions and thus may affect the ability of leukocytes to transmigrate.

Several PTPs have been shown to localize to junctions as well as interact with members of the cadherin-catenin complex (235). Specifically, PTPμ, DEP-1, PTP1B, and VE-PTP are found in endothelial cell junctions (81, 87, 90, 258). Previous studies in our lab revealed that leukocyte transmigration increased VE-cadherin phosphorylation at residues specific for p120ctn and β-catenin binding (259). When these residues were mutated to phenylalanine, transmigration was reduced, directly indicating the involvement of tyrosine phosphorylation of junctional proteins during leukocyte TEM (259). Several junctional binding partners for DEP-1 have been discovered such as β-catenin, plakoglobin and p120-catenin (80) and now occludin and ZO-1. Our preliminary data demonstrates that VE-cadherin may also be a substrate for DEP-1. With numerous junctional proteins as

substrates, it is likely that DEP-1 is involved in modulating phosphorylation of endothelial junctional proteins. Using the same phospho-specific antibodies used by Allingham et al.(259), we plan to explore whether DEP-1 interacts with the phosphorylated VE-cadherin residues necessary for catenin binding. We also plan to evaluate the effect that DEP-1 expression has on leukocyte TEM, and the phosphorylation of other substrates during the process of transmigration.

One manner by which DEP-1 activity might be altered during TEM is through binding of its extracellular domain. Receptor PTPs are proposed to act as adhesion receptors, transducing outside-in signaling. Initially we were interested in exploring the extracellular binding partners of DEP-1, especially those involved with leukocyte transendothelial migration (TEM). One possible interaction would be a homophilic, trans interaction of DEP-1 on adjacent endothelial cells. Other RPTPs are known to interact in homophilic interactions (84, 96, 120), which stabilize the protein at cell-cell contacts (94). The clustering of RPTPs at the plasma membrane brings the catalytic domain into close proximity with junctional protein substrates, promoting a net tyrosine dephosphorylation and potential strengthening of junctional integrity (78, 80, 94). Another possibility is that DEP-1 is interacting with itself on a migrating leukocyte. Hematopoietic cells express varying amounts of DEP-1 (CD148) (165-167), with white blood cells, in particular monocytes and macrophages, expressing very high levels of DEP-1 (165, 166). Therefore, DEP-1 on endothelial cells could be binding to DEP-1 on leukocytes and this interaction could affect the catalytic activity of DEP-1, altering phosphorylation of junctional proteins. A third hypothesis is that DEP-1 can form heterophilic interactions with other cell adhesion molecules on endothelium, leukocytes or both. JAMs are not exclusively found on cells that

form TJs, but are also on leukocytes (182). As a result, the extracellular domain of JAMs might interact with the extracellular domain of DEP-1 and aid in controlling TEM. Other potential targets are the extracellular domains of cadherins and growth factor receptors.

A number of extracellular ligands have been identified for a few RPTPs, including homophilic and heterophilic interactions (260, 261), however, ligands have not been identified for the majority of RPTPs. Although no extracellular ligands for DEP-1 have been identified yet, a recent study suggests that an undefined ligand is found in Matrigel, a commercial ECM protein preparation (100). Association of DEP-1 with Matrigel increased the intracellular phosphatase activity of DEP-1 (100). In addition, antibody-induced ectodomain oligomeriztion of DEP-1 also increased its catalytic activity (150). Therefore, interaction of DEP-1's extracellular domain with itself or binding an extracellular protein may result in an increase in PTP activity. In the model of leukocyte transmigration, increased activity of DEP-1 due to interactions with proteins on adjacent endothelial cells would allow for the dephosphorylation of junctional proteins, reformation of TJ and AJ complexes, and enhanced barrier function of endothelial cells. On the other hand, we would expect that binding to a leukocyte protein would inhibit PTP activity, allowing for increased phosphorylation of AJ and TJ proteins and loss of junctional integrity. It is possible that such a ligand exists and is awaiting discovery. Other attractive PTPs canditates for a role in leukocyte TEM are PTPμ and the vascular endothelial specific VE-PTP.

The Importance of RPTP Extracellular Domains

Signal transduction from RTKs emanates from the ligand binding extracellular domain. Ligand binding induces dimerization and autophosphorylation of the intracellular

tyrosines leading to activation and recruitment of downstream targets (262). In contrast, mechanisms controlling RPTP enzyme activity are unknown (263). RPTPs have extracellular domains that resemble other known cell adhesion molecules and it has been proposed that they can be involved in homo- and heterotypic intercellular binding (125). As stated in the introduction, RPTPs have traditionally been considered to be active as monomers and inactive when dimerized (125). This is known to be true for RPTPα (127-129), CD45 (264), SAP-1 (265), and PTPBR7 and PTP-SL (266). Other PTPs are able to dimerize, but it is unclear whether this dimerization affects their catalytic activity. In most cases, dimerization is independent of the catalytic domain and occurs through the extracellular domain or transmembrane domain. In fact, the transmembrane domain of PTPλ, PTP LAR, PTPα, PTPγ, GLEPP1, and DEP-1 are able to homodimerize in *Escherichia coli* membranes, albeit to different extents (267). In addition, several studies in eukaryotic cells have demonstrated that the transmembrane domain of PTPα is necessary and sufficient for mediating receptor dimerization (127, 129, 268). Furthermore, artificial induction of dimerization affects the enzyme activity of CD45, RPTPα, and DEP-1 (128, 150, 264).

Inhibition of the catalytic activity of PTPα has been proposed to occur by the catalytic domain of one PTPα being blocked by specific contacts with an inhibitory wedge domain from the membrane proximal region of the other PTPα partner (269). The residues that compose this structure in PTPα are conserved among other RPTPs including PTPμ and PTP-LAR (130, 131). However, structural analysis of the membrane-proximal catalytic domain of PTPμ and the entire cytoplasmic domain of PTP-LAR reveal that the inhibitory interaction between the catalytic domain and the wedge are not observed (130, 131). It remains to be determined whether the wedge domain can interact with the catalytic domain of other RPTPs,

proposing the possibility that this method of dimerization-induced inhibition may not be a universal mechanism regulating RPTP activity.

Interestingly, introduction of a cysteine residue in the extracellular juxtamembrane domain of RPTPα results in disulfide bond formation and dimerization (128). Depending on the exact location of the cysteines, the dimers are either active or inactive (128). This suggests that the orientation in which the extracellular domain dimerizes affects the intracellular orientation of the PTP domain and can influence its activity. SAP-1 is a RPTP which naturally contains an extracellular domain with several cysteine residues capable of forming disulfide bonds. Altering the redox state of the SAP-1 can reversibly control its dimerization and its activity (265). Moreover, PTPσ has two major isoforms, differing slightly in the number of their FNIII repeats. The alternative arrangements of the extracellular domain alters the ligand binding specificities of this PTP (270), potentially by changing the rotational conformation of the N-terminal extracellular domain. It is also possible that modifications of the cytoplasmic domains, such as phosphorylation or oxidation, also results in changes in the rotational coupling within RPTP dimers and alters ligand-binding properties. RPTPα-D2 (catalytic domain 2) changes its conformation in response to the cellular redox state of the cell (268), implying RPTPs may not only have the capacity for outside-in signaling but also inside-out signaling (261). Collectively, subtle changes in the relative orientation of RPTP dimers in both the extracellular and intracellular domains may be able to determine whether the PTP is active or inactive, providing a new mechanism for regulation.

The increasing complexity of factors that affect dimerization forces us to look at RPTP dimerization, ligand binding and activity more closely. How exactly are RTPs being

turned on and off? Perhaps the answer will never be as simple as it is for RTKs. Nevertheless, the extracellular domains of PTPs appear to be very important in determining their activity and downstream signaling pathways. Does the extracellular domain of a RPTP dictate whether dimerization activates or inhibits it? Or does the dimerization state of RPTPs affect their ability to bind to extracellular ligands, adding yet another level of complexity? Too many questions are left unanswered for us to clearly state how RPTP activity is regulated. In the end, the role of the extracellular domain may be PTP specific or even cell type specific, determined by the availability of ligands and signaling partners.

PTPs in Cancer

Cell adhesion plays a critical role in the process of metastatic tumor dissemination. Loss of cadherins expression and other cell adhesion molecules is a trademark sign of metastatic cells (271). Tumor development is associated with remodeling of the both TJs and AJs. Neoplastic cells escape from the constraints imposed on them by intercellular junctions and adopt a migratory behavior. Increased phosphorylation at junctions leads to disassembly of AJ and TJ protein complexes as well as decreased junctional integrity. Thus, PTP activity may influence invasion and metastasis in certain cancers.

Loss of function mutations

Loss of function of PTPs has been associated with cancer progression. Less than one third of point mutations in PTPs occur within the PTP domain, suggesting that loss of function is indirectly related to the structure of the catalytic domain (272). For example, RPTPρ is frequently mutated in human cancers including lung, colon, and skin (273). In colorectal tumor isolates, mutations were found in both the intracellular and extracellular

domains (273). Intracellular mutations diminished PTP activity as expected and it was proposed that mutations in the extracellular domain may alter protein-protein interactions (273). Five mutations were mapped to residues located in the MAM and immunoglobulin domains of RPTPρ, an area necessary for adhesion in PTPμ (274-276). Subsequently, these mutations were introduced into SF9 cells and impaired the homophilic interaction of PTPρ as demonstrated by the reduction of cell aggregate formation. This suggests that the mutations found naturally occurring in cancers were loss of function mutations (277). Disruption of protein-protein interactions may affect several signaling pathways and promote tumor migration and metastasis. A number of mutations in the DEP-1 gene, PTPRJ, also create coding polymorphisms and have been found in human cancers. Mutations in the extracellular domain are located in the 2^{nd} and 8^{th} fibronectin repeats (Fig. 1.4) (142, 163). These mutations are predicted to occur in exposed regions available for protein-protein interactions and could affect ligand binding, dimizeration, and downstream signaling pathways (163). Currently, the effects of these mutations have not been analyzed but it would be of interest to express them and compare effects of junctional integrity to wildtype DEP-1. Additionally, point mutations located in the proline-rich domain of PTP-PEST have been found in breast cancer cell lines (278). Two of these mutations enhance PTP catalytic activity whereas the third mutation inhibits activity (278). Shp-2 is yet another PTP in which mutations are associated with certain cancers including juvenile myelomonocytic leukemia, lung and colon cancer (279, 280). Several mutations in the N-terminal SH2 domain disrupt the inhibitory interaction with its catalytic domain, leading to activation of Shp-2 (281, 282). Therefore, mutations in non catalytic domains can both activate and

inhibit PTP activity, whether through altering localization or interrupting inter- or intramolecular interactions.

Loss of protein expression

In addition to loss of function mutations, loss of PTP expression is observed in several cancers. SAP-1 levels were down-regulated in advanced human hepatocellular carcinoma and (283). Protein expression correlated with the differentiation state of the cells, with normal tissue or well-differentiated HCC expressing similar or higher levels than non-cancerous tissue, and poorly differentiated HCC having reduced levels of SAP-1 (283). DEP-1 expression has been shown to be drastically reduced in multiple cancer cell lines and human cancers including thyroid, breast, pancreatic and colon cancers (139-142, 164). In addition, LOH of locus for the DEP-1 gene is frequent in human cancers (163). Therefore, DEP-1 has been proposed to act as a tumor suppressor by inhibiting cell proliferation. Studies have re-expressed DEP-1 in cancer cell lines lacking endogenous DEP-1 and found that there was a profound inhibition in cell proliferation (139-142, 164).

Besides controlling proliferation, DEP-1 may be inhibiting metastasis by reducing cell motility, as shown in Chapter 4, by means of regulating integrin-mediated signaling pathways as well as stabilizing cell-cell junctions through dephosphorylation of tight junction proteins (Chapter 2). Supported by other cancer models such as colon, pancreatic, and thyroid cancer (140-142, 164), we proposed that breast cancer cell lines would have reduced DEP-1 levels as well. The protein expression levels of DEP-1 were examined in normal-like breast epithelial cells (MCF10A) as well as dedifferentiated breast cancer cells (MCF7) and metastatic breast cancer cells (MDA-MB-231). Cells were lysed and total protein concentrations were normalized. Lysates were immunoblotted for DEP-1 and junctional

proteins β-catenin and E-cadherin. As seen in Fig. 5.1, DEP-1 protein levels increased as junctional proteins decreased. We anticipated seeing a decrease in junctional proteins in the metastatic cells, yet we hypothesized that DEP-1 levels would also decrease. One explanation for our observed increased in DEP-1 is that the cell's feedback machinery is trying to counterbalance overgrowth by DEP-1 expression, but perhaps another signaling pathway is overriding it. Another explanation is that the DEP-1 expressed in the MDA-MB-231 has disregulated activity possibly due to one of the point mutations found in cancer (163). To try and determine whether this was a possibility, we compared the catalytic activity of DEP-1 from MCF10A and MDA-MB-231 cells. In this preliminary experiment, DEP-1 was immunoprecipitated from an equal amount of total protein from both cell types. Half of the precipitated protein was run on a gel to determine the amount of DEP-1 immunoprecipitated and the other half was used in a pNPP assay. Significantly more DEP-1 immunoprecipitated from the MDA-MB-231 cells compared to the MCF10A cells (Fig. 5.2A). Interestingly, the catalytic activity of DEP-1 from the MCF10A cells was higher than the metastatic cells (Fig. 5.2B). Considering that substantially more DEP-1 was immunoprecipitated and thus used in the MDA-MB-231 activity assay, we can conclude that DEP-1 in the MDA-MB-231 cells may have a considerable reduction in catalytic activity. We plan to repeat this as well as to examine mRNA levels of DEP-1 in these cell types to determine whether transcription is altered. It would also be interesting to compare substrates for DEP-1 in normal versus breast cancer cell lines.

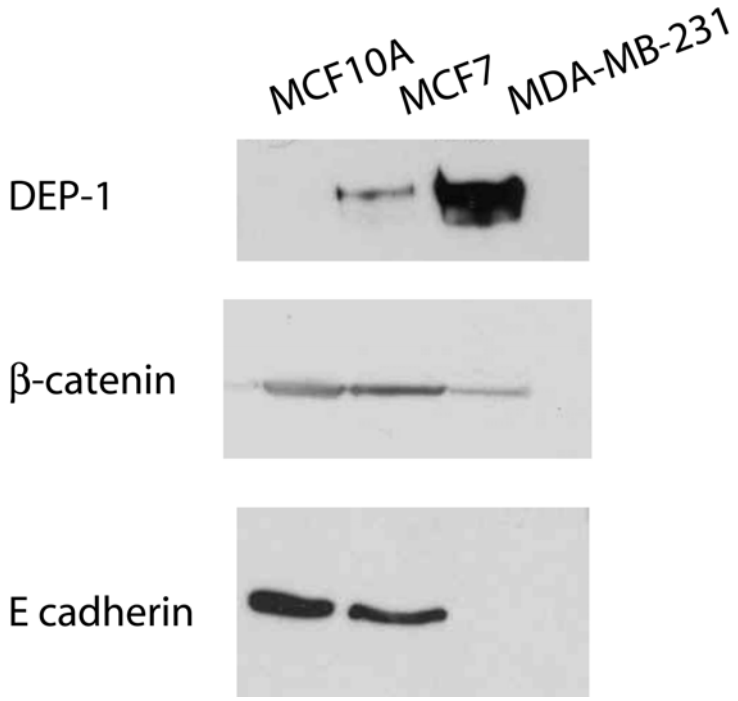

Figure 5.1. DEP-1 protein levels are increased in breast cancer cells compared to normal-like breast epithelial cells. Equalized total cell lysate from MCF10A, MCF7, and MDA-MB-231was immunoblotted with antibodies against DEP-1, β-catenin, and E-cadherin.

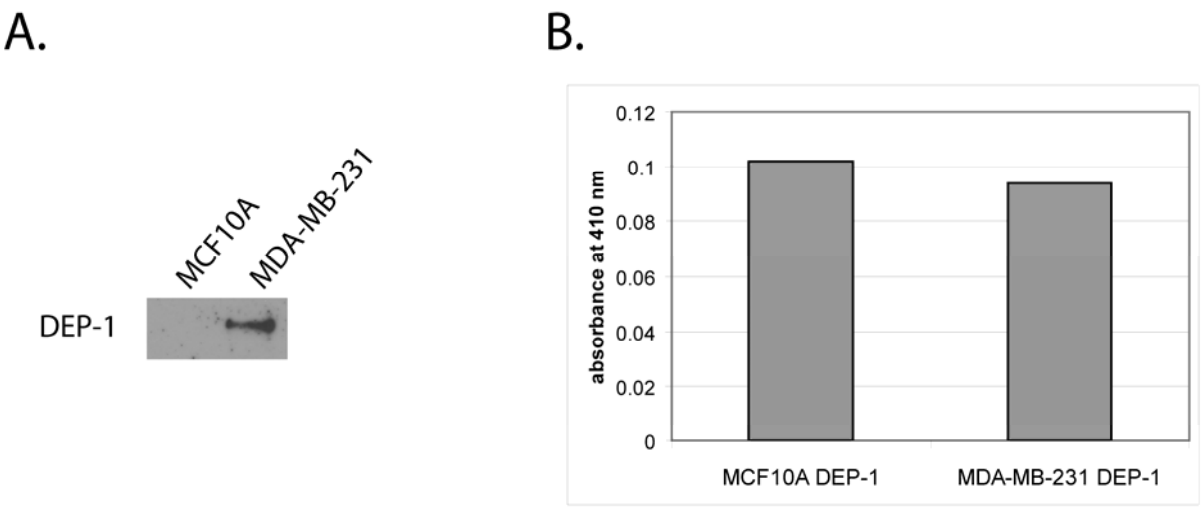

Figure 5.2. DEP-1 from breast cancer cells has decreased PTP activity compared to normal breast epithelial cells. DEP-1 was immunoprecipitated from both MCF10A and MDA-MB-231. The immunoprecipiation was divided in two and either **A.** run on a gel and immunoblotted for DEP-1 or **B.** used in a pNPP activity assay.

Over expression of DEP-1 in Cancer

With our result of increased DEP-1 in breast cancer cells, we were curious if other PTPs may be up regulated in cancers. One study found an increase in total PTP activity in breast cancer (284). Another group has shown that DEP-1 levels can vary in breast cancer cells lines, with MDA-MB-231 being one of the cell lines which exhibited the highest expression of DEP-1 (227). SAP-1 is overexpressed in human colorectal cancer (285) while PTP1B overexpression was established in breast tumor tissue (286). In addition, PTP-LAR mRNA and protein levels were markedly increased in breast cancer cell lines and tissues (287) while another study found that one third of the primary breast carcinomas tested were characterized by strongly eleveated RPTPα levels (288). Interestingly, these high protein levels were associated with low tumor grade (ER positive) and cells experimentally induced to have high PTPα cells showed delayed tumor growth and metastasis in mice (288). Increased PTP-LAR and PTP1B levels were found in human breast cancer as a consequence of expression of the activated neu oncogene. The amount of the PTP directly correlated to the expression of neu (289). Likewise, knockdown of PTPα induced apoptosis in ER negative but not ER positive breast cancer cells (290). These results insinuate that the genetic background of the cancer may regulate the expression level of the PTP as well as the signaling pathways in which the PTP is involved. Further studies will need to confirm this but it proposes an intriguing possibility.

PTP inhibitors

Over the last several years, PTP inhibitors have begun to be developed. Researchers are striving for a highly potent, yet selective small molecular inhibitor. Two main concerns

exist. First, PTPs contain a highly conserved active site, therefore obtaining inhibitors that selectively targets one PTP will be difficult. Second, a large number of side effects could occur because a single PTP might regulate multiple signaling pathways or a key pathway might be regulated by several PTPs (291). To address the first concern, researchers are looking for secondary binding sites in regions adjacent to the catalytic domain. Perhaps inhibitors targeted to these areas will enhance the inhibitors affinity and selectivity.

There are several PTP1B inhibitors currently being produced. PTP1B counteracts signaling through the insulin receptor (292), making a small molecule inhibitor of PTP1B a promising treatment for type 2 diabetes. Recently, mice from the well- established breast cancer mouse model (NDL2) were crossed with PTP1B deficient mice. Mice lacking PTP1B in this background displayed a reduced rate of tumor development, number of tumors, and lung metastases (293). Treatment of PTP1B +/+ mice with a PTP1B specific inhibitor also delayed the onset of mammary tumors (293). Consequently, PTP1B inhibitors might also used in cancer treatment, especially in ErbB2-positive breast cancers. Shp-2 has also been identified an oncogenic PTP and small molecule inhibitors are currently being developed (294). Although progress is being made for each of these PTPs, inhibitors for PTPs are still far behind the RTK inhibitor field.

For RPTPs, an alternative to interfering with the catalytic domain would be to target the extracellular domain and control activity by ligand-ectodomain interactions (137). The structural diversity of the extracellular domain makes it an attractive target to develop specific inhibitors. Antibodies to CD45 extracellular domain have already been shown to have the potential to inhibit CD45 activity (295, 296). Antibodies to additional PTPs may have a similar effect. Perhaps a compound that stabilized or locked the dimerization state

would prevent activity. It would be appealing to develop an inhibitor that would block the binding of a particular ligand and specifically affect downstream signaling.

Conclusion

The PTP field is experiencing an exciting time. Long gone is the stigma that PTPs are just "housekeepers" cleaning up after PTK signaling. PTPs are now considered to be as complex a group of proteins as PTKs, with activity regulated in specific ways. With the classification of all PTP genes in mice and humans (2), we can ask more questions regarding the specificity, redundancy, tissue expression and isoforms/mutations of PTPs. The development of the substrate trapping mutants has led to the identification of more substrates. Structural information regarding the extracellular and intracellular domains of PTPs is shedding new light on how catalytic activities are regulated. Now the field needs to focus on the physiological roles or functions of individual PTPs. One challenge facing researchers is that PTP expression and function may be regulated in a tissue-specific manner. In which case, different substrates might be identified in different cell types, resulting in modification of current signaling pathway models. Several PTPs have been linked to disease states such as diabetes, cancer and inflammation. Knockout mouse models, siRNA, and the development of small molecule inhibitors will help to clarify the roles of PTPs in normal physiological processes and pathological conditions. The future in PTP research possesses many discoveries for the years to come.

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